REMARKS

In an Office Action dated August 25, 2004, claims 15-16 and 18-26, all of the claims under consideration in the subject patent application, were rejected. By amendment above, claims 15 and 18 have been rewritten, claim 20 has been cancelled and claims 27 and 28 have been added. Support for the amendment to claims 15 and 18 can be found in claim 9 as originally filed, on page 7, line 12 to page 8, line 14 of the specification. Support for new claims 27 and 28 can be found on page 10, lines 3 to 5 of the specification. On page 26 of the specification a correction was made in table 8 to correct a typographical error. Support for the definition of groups 4 and 5 of yellowtails can be found in table 7 on page 26 of the specification. No new matter was added.

Reconsideration of this application and allowance of the claims is respectfully requested in view of the foregoing amendments and the following remarks.

The rejection of claims 15 and 16 under 35 U.S.C. 102(b) as anticipated by U.S. patent No. 5,494,819 issued to Soma et al. was maintained. The Examiner maintained his assertion that these claims are drawn to a product prepared from gram negative bacteria, having a molecular weight of 5000 ± 2000 as measured by the SDS-PAGE method, which product is also taught by Soma et al (US 5,494,819). The intended use as a feed composition for crustaceans and fish does not impart any critical impact or weight on the physical preparation and the patentability of the product, in the Examiner's view. According to the Examiner, Soma et al teach three products prepared from gram negative bacteria, having a molecular weights of 5000 ± 1000 and 6500 ± 2500 as measured by the SDS-PAGE method. The Examiner further asserts that these products are low molecular weight lipopolysaccharides (LMW-LPS) capable of activating immunity,

which can be used as feed or feed additive for veterinary use. According to the Examiner, Soma et al teach that the LPS is a 96% pure LPS with the dominant molecular weight of 5000 ± 1000 by SDS-PAGE analysis, wherein one of the LPS's is produced by a strain of species Pantoea agglomerans.

The Examiner has asserted that applicants' previous arguments are not persuasive. Applicants argued that Soma et al disclose a LPS composition which is partially purified, also containing high molecular weight LPS. Further, applicants argued that the LPS feedstuff composition of the present invention is further purified by removing HMW-LPS. Applicants submited that claim 15, which was amended to include the limitation "wherein the high molecular weight LPS is removed", should therefore not be considered anticipated by Soma et al as the HMW LPS is no longer present. In response, the Examiner asserts that the rejected claims are drawn to the same product as taught by Soma et al. According to the Examiner, Soma et al teach 96% purity for the product and thus the product of Soma et al can be considered 97%, 98%, 99%, or even 100% pure. Further, the Examiner asserts that the level of purity of LMW-LPS in the present claims is not clear. In addition, the term "removed" in "wherein the high molecular weight LPS is removed" can be interpreted as a removal of 100% or less of the high molecular weight LPS according to the Examiner.

Applicants submit that the present invention is directed to a feed composition for crustaceans or fish comprising as a feed-stuff additive highly purified LMW-LPS and to a method of activating immunity using the feed stuff additive. As is described on page 8, lines 1-14 of the specification, the present LMW-LPS is obtained by subjecting the LPS obtained by Soma et al. (U.S. 5,494,819, which is JP-A-4-99481), to gel filtration in the presence of a

surface-active agent to recover only low molecular weight LPS-containing fractions wherein the high molecular weight LPS is completely removed. Further, applicants amended claim15 to more clearly define the feed-stuff additive wherein the HMW-LPS is completely removed.

Applicants submit that Soma et al disclose a lipopolysaccharide (LPS) composition which is partially purified, also containing high molecular weight LPS, as a feed or feed additive. For example, Fig. 1 of the specification of Soma et al. illustrates that the LPS disclosed also contains HMW-LPS as HMW-LPS is clearly indicated in Fig. 1. In contrast to Soma et al, in the present invention the feed-stuff additive LMW-LPS is further purified by removing HMW-LPS. Therefore, the feed-stuff additive LMW-LPS of claim 15, as amended, is different from the LPS material in Soma et al, because the LPS material of Soma et al is further purified in the present invention to no longer contain any HMW-LPS.

Therefore, the invention as presently claimed is substantially different from the LPS material of Soma et al as not all limitations of the presently claimed invention are disclosed in Soma et al. Applicants respectfully submit that the presently claimed invention of independent claim 15 and dependent claim 16, is not anticipated by Soma et al (US 5,494,819). Withdrawal of the rejection is respectfully requested.

The Examiner also maintained his rejection of claims 18-26 under 35 U.S.C. 103(a) as being unpatentable over Takahashi et al (US Patent No. 5,641,761) in view of Soma et al (US Patent No. 5,494,819). According to the Examiner the claims are drawn to a method of activating immunity or preventing infection in crustaceans and fish comprising administering an effective amount of LMW-LPS to crustaceans and fish. The Examiner asserts that Takahashi et al teach a method of activating immunity, preventing infection in crustaceans, or treating

crustacean infections comprising administering or feeding a polysaccharide to crustaceans. The Examiner acknowledges that Takahashi et al do not teach the use of LMW-LPS. However, the Examiner asserts that because Soma et al teach a LMW-LPS capable of activating immunity as a feed or feed additive for veterinary use, it would have been have been obvious to modify the method of Takahashi et al by using the product of Soma et al to obtain the disclosed invention. According to the Examiner one would have been motivated to replace the HMW-LPS of Takahashi et al with the LMW-LPS of Soma et al which has excellent immuno-stimulating activity and may be provided at low cost and in a large amount.

According to the Examiner, applicants' argument, that a polysaccharide activating immunity in one animal does not confirm the same effect in crustaceans and Soma et al merely teaches LMW-LPS in feed for veterinary use, is not persuasive. According to the Examiner, Takahashi et al recite that their polysaccharide shows effects for preventing infectious diseases and enhancing the immune system of fish and crustaceans. Further, the Examiner asserts that one of ordinary skill in the art would be motivated to combine the teachings of Takahashi et al (teaching that polysaccharides enhance immune system of fish and crustaceans) with Soma et al (teaching a LMW-LPS from bacteria which has excellent immuno-stimulating activity). In addition, the Examiner asserts that limitations such as concentration of the feed are being viewed as limitations of optimizing experimental parameters.

Applicants submit that claims 18-26 are non-obviousness because the use of the specific Gram-negative bacteria of the genus Pantoea in obtaining LMW-LPS unexpectedly resulted in a feed stuff additive useful in increasing immunity in crustaceans and fish. Lipopolysaccharides (LPS) of a low molecular weight do not necessarily have high biological activity depending on

the type of lipopolysaccharide used. Many different lipopolysaccharides (LPS) of low molecular weight (LMW-LPS) are described in Soma et al (USP 5,494,819). It is not obvious that the specific LMW-LPS of the present invention obtained by use of the Gram-negative bacteria of the genus Pantoea exhibits high biological activity and excellent effects as an additive for crustacean or fish feed.

Rau et al (Arch. Microbiol. 1995, 164: 280-289 disclose on page 287 that "LPS of *R. salinarum* had a lethality for mice (C57BL/10ScSN mice) of 1/10 to 1/100 compared with that reported for *Salmonella abortus equi* LPS (LD_{50} between 0.01 μg and 0.001 μg). Further, *R. salinarum* LPS also induced TNF-α and IL-6 generation in macrophages. In these in vitro test systems (data not shown), LPS of *R. salinarum* had 10% the activity of that of *Salmonella abortus equi* LPS." *R. salinarum* LPS has low molecular weight similar to *Salmonella minnesota* R595 as shown on page 282, Fig 1, lanes 7 and 9 of Rau et al. *Salmonella minnesota* R595 is commercially available and is well known to have molecular weight of about 2700. In contrast, LPS of *Salmonella abortus equi* has a high molecular weight of more than 20000.

LPS consist of a lipid A, a core polysaccharide and O-specific chains. These O-specific chains have repeated units of various oligosaccharides. It is well known that types of LPS differ depending on these oligosaccharides and number of repeating units. The electrophoretic pattern of LPS of *Salmonella abortus equi* is discloses on page 401, Fig. 1, lane 1 of Galanos et al (J. of Chromatography 1988, 440; 397-404). The reference further discloses that the Long-chain LPS (LPS of *Salmonella abortus equi)* consists of molecules with 20-50 repeating units in the O-polysaccharide part. It can be derived from the disclosure in Galanos et al (Bacterial Endotoxin; Chemical, Biological and Clinical Aspects: Verlag Chemie 1984: 409-413) that the molecular

weight of LPS of *Salmonella abortus equi* is about 24400 to 54400. The reference discloses on page 412 (Fig 2) that in *Salmonella abortus equi* LPS consists of a Lipid A having a molecular weight of about 2000, a core polysaccharide having a molecular weight of about 2400, and O-specific chains having a molecular weight of about 20000 to 50000.

Therefore, *Salmonella abortus equi* LPS, a LPS having a high molecular weight, shows a 10 times higher biological activity than the LPS of *R. salinarum*, a LPS having low molecular weight. Accordingly, LMW-LPS does not necessarily have increased biological activity depending on the type of lipopolysaccharide used.

Furthermore, Zahringer et al (J.Biol.Chem. 2004, 279: 21046-21054) discloses on page 21052, Fig. 7-A, that the purified *B. henselae* LPS induces production of 1000 ng/ml of IL-8 at a concentration of 100 ng/ml in vitro in HEK 293 cells. In contrast, *Legionella pneumophila* LPS induces almost same production of IL-8 at a concentration of 1 ng/ml. Thus, *B. henselae* LPS has 1/100 the activity of that of *Legionella pneumophila* LPS. LPS from *B. henselae*, a Gram negative bacterium, has molecular weight of about 2600 to 4000 as apparent from page 21048 of the reference. *Legionella pneumophila* LPS has molecular weight of about 30000 to 50000. See, Luneberg et al (J.Exp.Med. 1998, 188:49-60). Therefore, LMW-LPS does not necessarily have higher biological activity compared to HMW-LPS.

To further illustrate that a lower molecular weight of lipopolysaccharides does not correlate to high biological activity of lipopolysaccharide, the declaration by Prof. Genichiro Soma submitted herewith shows that this activity depends on the type of lipopolysaccharide. In other words, a lipopolysaccharide of a low molecular weight does not necessarily have a high biological activity. It is therefore not obvious that the specific LMW-LPS of the present

invention obtained from Gram-negative bacteria of the genus Pantoea exhibits high biological activity and excellent effects as an additive for crustacean or fish feed.

Takahashi et al teach the use of a particular polysaccharide in feed for crustaceans and fish to activate immunity, prevent infection and treat infections in crustaceans. Takahashi et al use a polysaccharide derived from mushrooms in contrast to the LMW-LPS of the present invention. Soma et al merely teaches that LMW-LPS in feed for veterinary use activates immunity in animals. However, Soma et al do not teach or suggest that the use of a specific LMW-LPS of the present invention, which is completely free of high molecular weight LPS, in feed for crustaceans and fish activates immunity.

Therefore, there is nothing in the disclosure of Takahashi et al and Soma et al which would lead the skilled person to arrive at the present invention as a matter of routine. Thus, combining Takahashi et al and Soma et al does not result in the use of a specific LMW-LPS of the present invention in a method of activating immunity, preventing and treating infections in crustaceans or fish. Thus, applicants respectfully submit that the presently claimed invention of claims 18-26, is not obvious over Takahashi et al (US 5,641,761) in view of Soma et al (US 5,494,819). Withdrawal of the rejection is respectfully requested.

The Examiner also rejected claims 15-26 under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which is regarded as the invention. According to the Examiner, claims 15 and 18 recite in step A "wherein the high molecular weight LPS is removed". The Examiner asserts that in direct contradiction with this term in step A is that step C recites that the product is "substantially free of high molecular weight LPS". Therefore, according to the Examiner it is not clear what the

degree of purity of the claimed product is. Claims 16 and 19-26 are indefinite as being dependent from claims 15 and 18.

To more clearly define the subject matter of the invention, applicants amended claims 15 and 18 amending the terms "wherein the high molecular weight LPS is removed" in step A and "wherein the LPS is substantially free of high molecular weight LPS" in step C to recite that the high molecular weight LPS is completely removed. Therefore, applicants submit that the product of the invention and its purity as in claims 15 and 18, and claims dependent thereon, is more clearly defined. Withdrawal of the rejection is respectfully requested.

Further, enclosed for the Examiner's convenience are the publications referenced to in this Amendment and Request for Reconsideration. Applicants submits that the present application is now in condition for allowance. Reconsideration and favorable action are earnestly requested.

RESPECTFULLY SUBMITTED,					
NAME AND REG. NUMBER	Willem F.C. de Weerd, Registration No. 51,613				
SIGNATURE				DATE	10/24/04
	Rothwell, Figg, Ernst & Manbeck 1425 K Street, N.W., Suite 800				
City	Washington	*State*	D.C.	*Zip Code*	20005
Country	U.S.A.	*Telephone*	202-783-6040	*Fax*	202-783-6031

Arch Microbiol (1995) 164:280–289

ORIGINAL PAPER

Heike Rau · Ulrich Seydel · Marina Freudenberg
Jürgen Weckesser · Hubert Mayer

Lipopolysaccharide of *Rhodospirillum salinarum* 40: structural studies on the core and lipid A region

Received: 12 May 1995 / Accepted: 2 July 1995

Abstract The structural elucidation of lipid A of the cell wall lipopolysaccharide (LPS) of *Rhodospirillum salinarum* 40 by chemical methods and laser desorption mass spectrometry revealed the presence of a mixed lipid A composed of three different 1,4′bisphosphorylated β(1→6)-linked backbone hexosaminyl-hexosamine disaccharides, i.e. those composed of GlcN→GlcN, 2,3-diamino-2,3-dideoxy-D-Glc-(DAG)→DAG, and DAG→GlcN. Lipid A of *R. salinarum* contained preferentially 3-OH-18:0 and 3-OH-14:0 as amide-linked and *cis*Δ^{11}–18:1 and c19:0 as ester-linked fatty acids. The mass spectra of the liberated acyl-oxyacyl residues proved the concomitant presence of 3-*O*-(*cis*Δ^{11}–18:1)–18:0 and 3-*O*-(c19:0)-14:0 as the predominating diesters in this mixed lipid A. The glycosidically linked and the ester-linked phosphate groups of the backbone disaccharide were neither substituted by ethanolamine, phosphorylethanolamine, nor by 4-amino-4-deoxy-L-arabinose, in contrast to most of the enterobacterial lipid As. In the core oligosaccharide fraction, a HexA (1→4)HexA(1→5)Kdo-trisaccharide was identified by methylation analysis. The terminal HexA (hexuronic acid) is possibly 4-OMe-GalA, a component described here as an LPS constituent for the first time. LPS of *R. salinarum* showed a lethality in C57BL/10 ScSN (LPS-responder)-mice) of an order of 10^{-1}–10^{-2} of that reported for *Salmonella abortus equi* LPS, and it was also capable of inducing TNFα and IL6 in macrophages of C57BL/10ScSN mice.

H. Rau · M. Freudenberg · H. Mayer (✉)
Max-Planck-Institut für Immunbiologie, Stübeweg 51,
D-79108 Freiburg i. Br., Germany
Tel. +49-761-5108227; Fax +49-761-5108221

U. Seydel
Forschungsinstitut Borstel, Parkallee 22, D-23845 Borstel, Germany

J. Weckesser
Institut für Biologie II, Mikrobiologie, Schänzlestrasse 1,
D-79104 Freiburg i. Br., Germany

Key words *Rhodospirillum salinarum* · Lipopolysaccharide · Mixed lipid A · 2,3-Diamino-2,3-dideoxy-D-glucose · 4-*O*-Methyl-galacturonic acid · Halophilic bacteria · Lethal toxicity

Abbreviations *AAS* Atomic absorption spectroscopy · *c19:0 cis*- 11,12 Methylene-octadecanoic acid · *DAG* 2,3-Diamino-2,3-dideoxy-D-glucose · *DMDS* Dimethyl-disulfide · *DOC-PAGE* Deoxycholate-polyacrylamide gel electrophoresis · *EI-MS* Electron impact mass spectrometry · *GalA* Galacturonic acid · *GlcA* Glucuronic acid · *GC-MS* Combined gas liquid chromatography-mass spectrometry · *GlcN* D-Glucosamine · *HexA* Hexuronic acid · *IL1* Interleukin 1 · *IL6* Interleukin 6 · *Kdo* 3-Deoxy-D-manno-octulosonate · *LD-MS* Laser desorption mass spectrometry · *LPS* Lipopolysaccharide · *MTT* 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide · *4-OMe*-GalA 4-*O*-methylgalacturonic acid · *PITC* Phenyl isothiocyanate · *TNFα* Tumor necrosis factor α

Introduction

Lipopolysaccharides (LPS) are amphiphilic glycoconjugates, consisting of a lipid component, lipid A, and a hydrophilic polysaccharide part, which is composed of the *O*-specific chain and the core oligosaccharide (Rietschel et al. 1992). Lipid A, responsible for manifold endotoxic properties and other biological activities (Galanos et al. 1985) induces mediators on macrophages, such as for example, tumor necrosis factor α (TNFα), interleukin 1 (IL1), and interleukin 6 (IL6). Their overproduction can result in endotoxic shock (Old 1988; Rietschel and Brade 1993). The lipid A region, although phylogenetically rather preserved, may show some structural variability and can thus be used in some cases as a chemotaxonomic marker to indicate phylogenetic relationships between bacterial species (Mayer et al. 1990a, b).

The moderate halophilic bacterium *Rhodospirillum salinarum* 40, used in this study is a red spirillum isolated

from a Portuguese saltern (Nissen and Dundas 1984) and is a member of the α-1 subgroup of Proteobacteria. This subgroup comprises additionally, for example, *Rhodospirillum fulvum, Aquaspirillum itersonii, Aquaspirillum polymorphum, Rhodopseudomonas globiformis,* and *Azospirillum brasilense* (Woese et al. 1984; Stackebrandt et al. 1988).

Materials and methods

Bacterial strain and cultivation

Rhodospirillum salinarum strain 40 was obtained from J. Imhoff (University of Kiel, Germany). Bacteria were grown anaerobically at 32° C in the light using the medium of Nissen and Dundas (1984) with 10% NaCl.

Preparation of LPS

Bacteria were pretreated with ethanol, acetone, and diethylether (Rau et al. 1994). Subsequently, LPS was extracted by the hot-phenol-water extraction procedure of Westphal and Jann (1965), followed by repeated (three times) centrifugation at 105 000 × *g* for 4 h each. The water phase/LPS was treated with ribonuclease. LPS was further purified by subsequent phenol/chloroform/petroleum ether extraction according to Galanos et al. (1979) and by extraction of the contaminating ornithine lipid by the "CMA" mixture (see below).

Preparation of lipid A and "degraded polysaccharide"

LPS was hydrolyzed (1% acetic acid, 100° C, 90 min) and the resulting lipid A and "degraded polysaccharide" fractions were further purified. After centrifugation (3000 × *g*, 30 min) lipid A was washed once with cold water, twice with 40° C-warm water, and finally with ethanol and acetone. After lyophilization, lipid A was dissolved in chloroform/methanol (9:1; v/v), and hydrophilic contaminants were removed by centrifugation (3000 × *g*, 20 min). The lipid A-fraction was precipitated by dropwise addition of water to this solution.

For laser desorption mass spectrometry (LD-MS), dephosphorylated and de-*O*-acylated lipid A was prepared by alkali treatment (0.5 M sodium methylate, 4° C, 24 h) of the underivatized lipid A. "Degraded polysaccharide" was lyophilized and used for the characterization of a core-derived acidic trisaccharide.

Extraction of ornithine lipid

The contaminating ornithine lipid was extracted from LPS using the "CMA" mixture (chloroform/methanol/7M ammonia; 65:25:4, by vol.) according to Holst et al. (1983).

Sodium deoxycholate-polyacrylamide gel electrophoresis (DOC-PAGE)

DOC-PAGE of isolated lipopolysaccharide was carried out according to Komuro and Galanos (1988). The gels were silver-stained after oxidation with periodic acid (Tsai and Frasch 1982).

Fatty acid analysis

Fatty acids, liberated by 1 M methanol-HCl (85° C, 16 h) as their methylester derivatives, were quantified by gas liquid chromatography-mass spectrometry (GC-MS) on a DB5MS (Fissons, Mainz-Kastell, Germany) capillary column (30 m, 0.25 mm i.d.), using the following temperature programm: 50° C/min between 100 and 180° C and 5° C/min between 180 and 240° C; injector temperature 250° C. Ester- and amide-bound fatty acids were analyzed separately by sodium methylate treatment (Wollenweber and Rietschel 1990) with the same column and temperature program as above.

Acyl-oxyacyl residues were liberated by a procedure of Wollenweber et al. (1982). The diesters were analyzed by GC-MS on a DB1 capillary column (30 m, 0.25 mm i.d.) using the following temperature programm: 3 min at 240° C, 5° C/min between 240 and 320° C; injector temperature 300° C.

After methanolysis (85° C, 18 h) and extraction of the fatty acid methyl esters, 100 µl dimethlydisulfide-solution (DMDS; 99% gold label, Aldrich, Steinheim, Germany) and 2 drops of iodine solution [6% (w/v) in diethylether] were added (Dunkelblum et al. 1985; Moss and Lambert-Fair 1989) and incubated for 6 days at 50° C. For extraction, 1 ml hexane and 1 ml 5% NaS_2O_3 solution were added to the sample. After extensive shaking and centrifugation, the fatty acid methyl esters and the DMDS-derivatives of the unsaturated fatty acid methyl esters were obtained in the organic phase. The sample was analyzed on an SP 2380 capillary column (30 m, 0.25 mm i.d.) (temperature program: 2 min at 50° C, 35° C/min between 50 and 170° C, and 10° C/min between 170 and 240° C; injector temperature 250° C).

Sugar analysis

For quantitative determination of neutral and amino sugars, hexuronic acids, and 3-deoxy-D-manno-octulosonic acid (Kdo) by GC-MS on a fused silica DB1 (Fissons) capillary column (30 m, 0.25 mm i.d.), the sample was treated according to the method of Russa et al. (1991). Briefly, the sample was hydrolyzed with 1% acetic acid (100° C, 1.5 h), reduced with $NaBH_4$, methanolyzed (0.5 M methanolic HCl, 85° C, 16 h), carboxy-reduced with $NaBD_4$ (4° C, 48 h), hydrolyzed with 1 M trifluoroacetic acid (120° C, 2 h), further reduced with $NaBD_4$, and finally peracetylated (100° C, 1 h) with pyridine/acetic anhydride. Amino sugars were released with 4 M HCl (100° C, 18 h) and identified by high-voltage paper electrophoresis (Kickhöfen and Warth 1968) in a pyridine/formic acid/acetic acid/water (1:1.5:10:90, by vol.; pH 2.8) buffer system and by HPLC of their phenyl isothiocyanate (PITC) derivatives on a Waters 860/V2.3 liquid chromatograph (Waters, Eschborn, Germany) using authentic sugar standards. Amino sugars were also quantified as alditol acetates (Lindberg 1972) by GC-MS on a DB1 capillary column (30 m, 0.25 mm i.d.) (temperature program: 50° C/min between 50 and 180° C, 4° C/min between 180 and 200° C, and 7.5° C/min between 200 and 260° C; injector temperature 280° C). In this case, amino sugars were *N*-acetylated prior to reduction by addition (three times) of 5 µl acetic anhydride and 5 µl 10% aqueous NaOH solution. On electropherograms, the amino sugars were visualized with 0.25% ninhydrin in acetone and silver nitrate. Alditol acetates and fatty acid methyl esters were identified and quantified by comparison with authentic internal standards and with reference substances (xylose, galactosamine, and heptadecanoic acid). Uronic acids were released by hydrolysis with 1 M trifluoracetic acid for 2 h at 120° C and identified by high-voltage paper electrophoresis in the above-mentioned buffer system.

Phosphorus

Total phosphorus was determined by the method of Lowry et al. (1954). ^{31}P NMR spectra (Rosner et al. 1979; Batley et al. 1985) were registered on a Bruker WM300 spectrometer at 121.51 MHz. Chemical shifts were measured relative to an external ^{31}P source (85% phosphoric acid; 0.00 ppm) at 33° C.

Laser desorption mass spectrometry (LD-MS)

LDMS analyses were performed on a Lamma 500 instrument (Leybold-Heraeus, Köln, Germany). Preparation of the samples and the details of analysis have been described elsewhere (Seydel et al. 1984 a, b). NaI was used for ionization by Na^+ attachment.

Reduction and methylation of "degraded polysaccharide"

After reduction, the "degraded polysaccharide" was methylated (Hakomori 1964; Lindberg 1972) and purified over a Sep-Pak-C_{18}-cartridge (Waters, Milford, Mass., USA) (Mort et al. 1983). The sample was analyzed by GC-MS on a DB1 capillary column (30 m, 0.25 mm i.d.) with the following temperature programm: 2 min at 80°C, 5°C/min between 80 and 300°C; injector temperature 270°C. The peak eluting at 40.5 min contained the HexA→HexA→Kdo trisaccharide.

Atomic absorption spectroscopy (AAS)

About 1 mg of the substance, one glass pearl, 1 ml concentrated nitric acid, and 0.4 ml perchloric acid were added to a Kjeldahl flask. The volume was then reduced to half by heating, and the samples were placed into a new flask containing 1 ml aqueous solution of 1% Cs salts and 10% lanthane salts. Subsequently, the flask was filled with water to a volume of 10 ml.

A Perkin-Elmer (Norwalk, Conn., USA) atom absorption spectrometer 280 was used at the following wavelengths according to the manual: Ca^{2+}: 422.7 nm; Mg^{2+}: 285.2 nm; Na^+: 589.0 nm; K^+: 766.5 nm; gas: C_2H_2, oxidant: air. The instrument was calibrated with standard solutions of known Ca^{2+}, Mg^{2+}, K^+, and Na^+ concentrations.

Lethal toxicity in mice

Various concentrations of LPS in 0.2 ml phosphate-buffered saline were injected together with 20 mg galactosamine into C57BL/10 ScSN mice. The number of dead mice was determined after 24 h. As a control, C57BL/10 ScCR-mice (LPS-"nonresponder" mice) were used.

Induction of TNFα and IL6 in macrophages

TNFα was induced in macrophages according to Freudenberg and Galanos (1991), and IL6 was induced in macrophages according to M. Freudenberg (unpublished material). Briefly, B9-cells, an IL6-dependent mouse tumor cell line, were used for the latter test. RPMI 1640-medium (plus 10% fetal calf serum, Biochrom, Berlin, Germany) was prepared at a concentration of 10^5 cells/ml. In microtiter plates (F96, Nunc, Roskilde, Denmark), 100 µl RPMI 1640 medium was dispensed, and 25 µl of supernatants of bone marrow macrophages was then added in dilutions (1:5). Bone marrow macrophages of both C57BL/10 ScSN and C57BL/10 ScCR mice were used. Finally, 100 µl cell suspension was added to each well. As a standard, recombinant IL6 (200000 U/ml, Boehringer, Mannheim, Germany) was used, and a medium control was performed. The microtiter plates were incubated at 37°C with 5% CO_2 for 4 days. Subsequently, 50 µl MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (Sigma, Deisenhofen, Germany); 1 mg/ml in Dulbecco's PBS, Amimed, Muttenz, Switzerland] was added to each well. The plates were incubated at 37°C and with 5% CO_2 for 4 h. Living cells were stained with MTT. The plates were centrifuged at 300 × *g* for 7 min in a minifuge, and the supernatants were removed by aspiration. Finally, 100 µl 0.06 M HC in isopropanol was added to each well. The plates were shaken for 10 min and then measured in an ELISA reader (SLT, Crailsheim, Germany) at a wavelength of 550 nm and at the reference wavelength (690 nm).

Results

Lipopolysaccharide extraction and DOC-PAGE

LPS of *Rhodospirillum salinarum* was obtained from the water phase of phenol/water extracts at a yield of 3.2%, based on bacterial dry weight. From the DOC-PAGE pattern, it was evident that the *R. salinarum* LPS consisted of two major fractions of R-type character together with two slower moving bands, the latter weakly indicating a ladder-like pattern (Fig. 1). The fastest-moving band with R-type character was similar to that of the Re-type of *Salmonella*, whereas the slower-moving band resembled that of LPS of the Rb_2-type of *Salmonella*.

Removal of ornithine lipid

On high-voltage paper electrophoresis of acidic hydrolysates, followed by ninhydrin staining, considerable amounts of ornithine (5.6%, quantified by HPLC) were detected in addition to D-glucosamine (GlcN) and 2,3-diamino-2,3-dideoxy-D-glucose (DAG). The existence of ornithine in the LPS fraction probably indicated a co-extracted or-

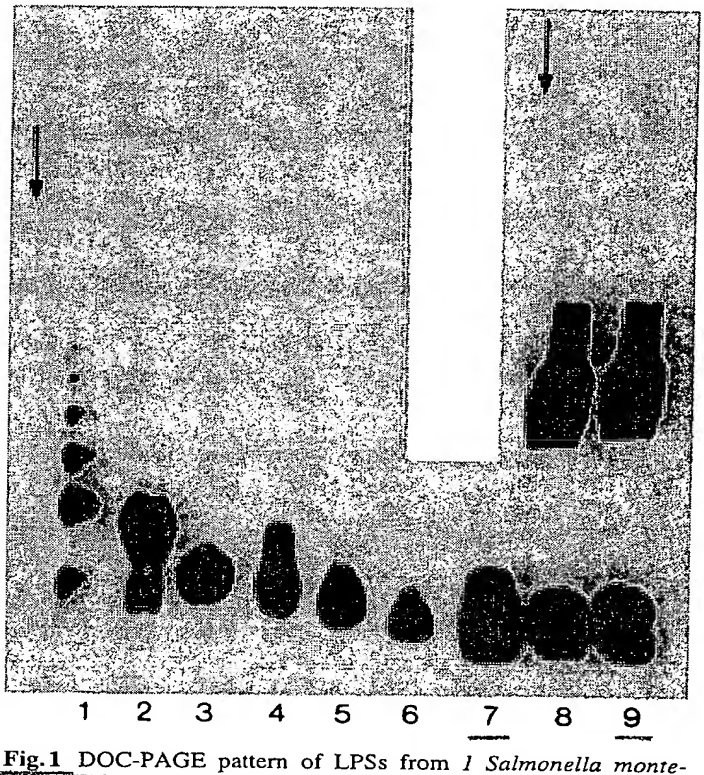


Fig. 1 DOC-PAGE pattern of LPSs from *1 Salmonella montevideo* SH94, 5 µg, *2 Salmonella typhimurium* SH777, 3 µg, *3 Salmonella djakarta* 10404, 2 µg, *4 Salmonella minnesota* R60, 6 µg, *5 Salmonella typhimurium* TV166, 6 µg (Rb_2-type), *6 Salmonella typhimurium* SL848, 5 µg (Rc-type) *7 Salmonella minnesota* R595, 5 µg (Re-type), *8 Rhodospirillum salinarum* strain 40, 10 µg, and *9 Rhodospirillum salinarum* strain 40, 15 µg. *Insert:* Samples from lanes 8 and 9 obtained with a gel containing 14% acrylamide

Table 1 Chemical analysis of the lipopolysaccharide (LPS) of *Rhodospirillum salinarum* strain 40. Values are given in μg/mg and nmol/mg LPS dry weight. [++ relative amounts of DAG that could not be quantitated with the method according to Russa et al. (1991) because of too mild hydrolytic conditions, *GLC* glucose, *Gal* galactose, *GlcA* glucuronic acid, *GalA* galacturonic acid, *4-OMe-GalA* 4-*O*-methyl-galacturonic acid, *LD-Hep* L-glycero-D-manno-heptose, *Kdo* 3-deoxy-D-manno-octulosonate, *DAG* 2,3-diamino-2,3-dideoxy-D-glucose]

Components	[μg/mg]	[nmol/mg]	[%]
Sugars			
Glc	106.3	590.6	10.6
Gal	192.2	1067.8	19.2
GlcA	42.5	219.1	4.2
GalA	307.6	1585.6	30.7
4-*O*Me-GalA	135.6	651.9	13.5
LD-Hep	42.0	200.0	4.2
Kdo	55.5	233.2	5.5
GlcN	29.0	162.0	2.9
DAG	++	++	++
Fatty acids			
16:0	9.0	35.2	0.9
cisΔ^{11}18:1	13.1	46.5	1.3
c19:0	5.0	16.9	0.5
3-OH-14:0	32.5	133.2	3.2
3-OH-16:0	2.2	8.1	0.2
3-OH-18:0	54.2	180.7	5.4
Phosphate			1.5

nithine lipid. It was separated from LPS by extraction with the "CMA"-mixture (chloroform/methanol/7M ammonia, 65:25:4, by vol.), at a yield of 2.7% based on LPS dry weight. It was not further investigated.

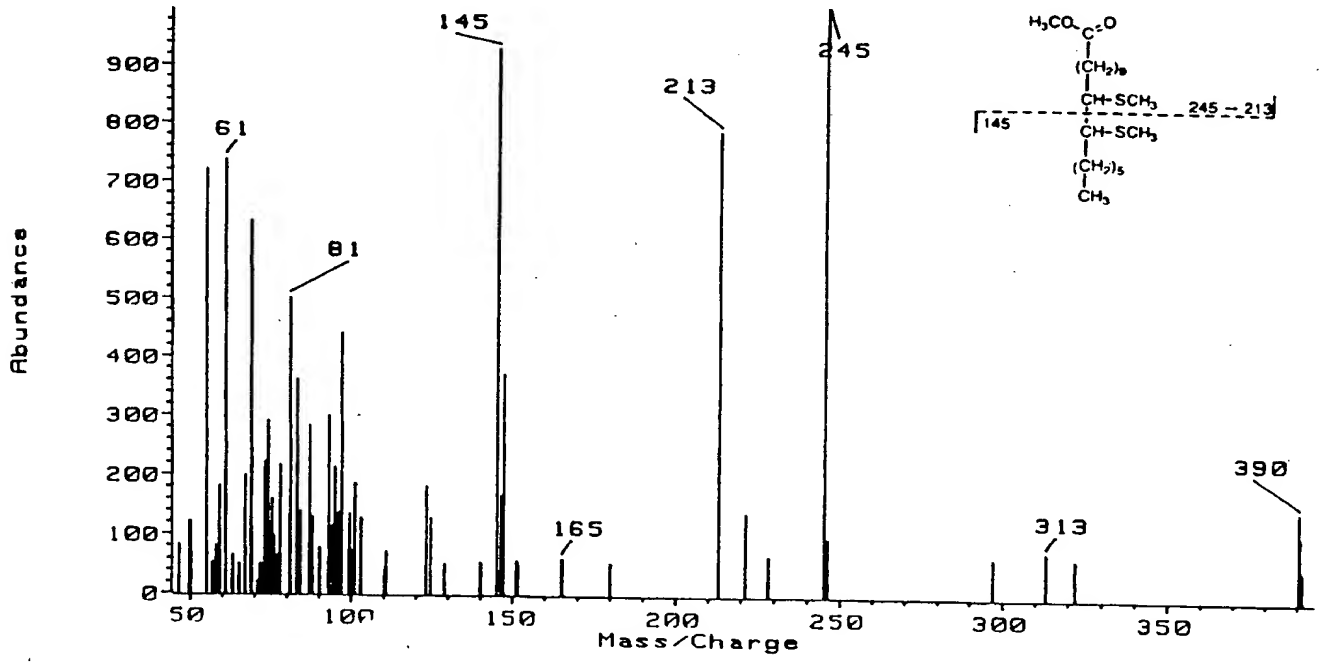
Fatty acid analyses

The total fatty acid content of the LPS amounted to 11.6% of LPS dry weight. The LPS was especially rich in hydroxy fatty acids (Table 1). The amounts of the two main hydroxylated fatty acids, 3-OH-18:0 and 3-OH-14:0, dominated over the non-hydroxylated fatty acids, 16:0, *cis*Δ^{11}-18:1, and c19:0 (molar ratio 3.25:1)(Table 1). This ratio indicated only smaller amounts of diesters in lipid A, in addition to unsubstituted 3-hydroxylated fatty acids.

The location of the double bond in 18:1 was revealed by DMDS derivatization (Fig. 2). The fragment m/z 245 [and m/z 213 (245–32)] represents the carboxy terminal half of the molecule, whereas the fragment m/z 145 represents the aliphatic end. With an authentic standard of 18:1, it was identified as *cis*Δ^{11}-18:1; c19:0 yielded a mass spectrum similar to 19:1, but was differentiated from 19:1 by treatment with DMDS. Only 19:1, but not c19:0, formed a DMDS derivative, indicating the presence of the latter in the lipid A moiety.

All of the 3-OH-18:0 was found to be amide-linked and involved in the 3-acyl-oxyacylester formation with *cis*Δ^{11}-18:1. The 3-hydroxymyristic acid was amide-linked and ester-linked and was partially substituted by c19:0. The mass spectra and fragmentation schemes of 3-*O*-(c19:0)-14:0 and 3-*O*-(*cis*Δ^{11}-18:1)-18:0 are shown in Fig. 3. The peaks were identified by comparing the characteristic fragments originating from the 3-hydroxyacyl groups and from the substituents of the 3-hydroxyacyl group. The characteristic fragments obtained from 3-*O*-(X)-14:0

Fig. 2 Electron impact mass spectrum and fragmentation scheme of the dimethyldisulfide-derivative fatty acid methylester of *cis*Δ^{11}18:1 from the *Rhodospirillum salinarum* lipopolysaccharide (LPS)



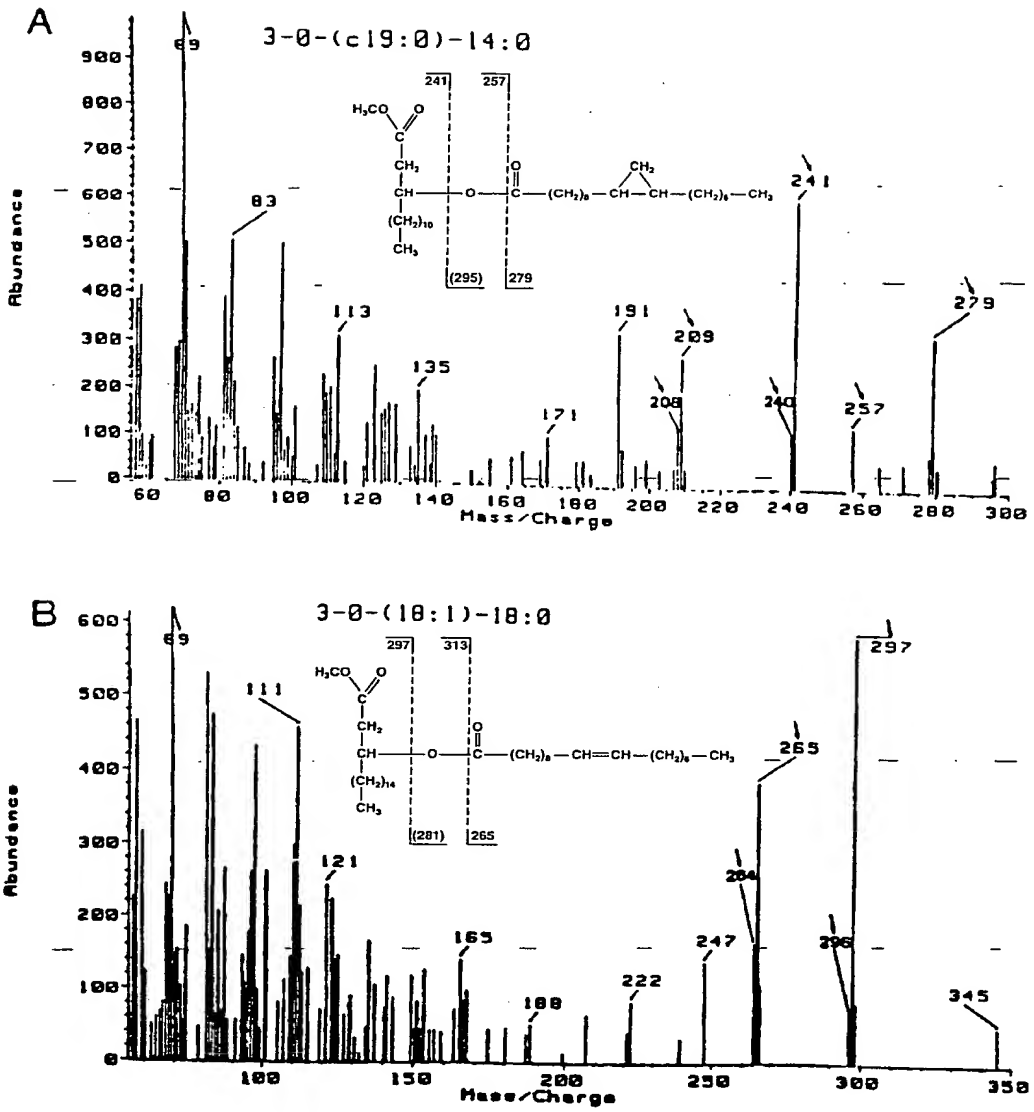


Fig. 3 Mass spectra and fragmentation schemes of A the methylester 3-*O*-(c19:0)-14:0 and B 3-*O*-(*cis*Δ^{11}18:1)-18:0 obtained from CH_3I/Ag^+-treated *Rhodospirillum salinarum* free lipid A. The *arrows* indicate the characteristic fragments for A the 3-hydroxyacyl group 3-*O*-(X)-14:0 (m/z 208, 209, 240, 241, 257) and the substitutent of the 3-hydroxyacyl group c19:0 (m/z 279) and B the 3-hydroxyacyl group 3-*O*-(X)-18:0 (m/z 264, 265, 296, 297, 313) and the substituent of the 3-hydroxyacyl group *cis*Δ^{11}18:1 (m/z 265)

were m/z 208, 209, 240, 257 (Fig. 3 A), whereas the diagnostic fragment of the substituent (c19:0) of the 3-hydroxy group was m/z 279. In the case of the diester 3-*O*-(*cis*Δ^{11}-18:1)-18:0, m/z 264, 265, 296, 297, and 313 characterized the 3-hydroxyacyl-group and the fragment m/z 265 characterized the ester-bound substituent, i.e., *cis*Δ^{11}-18:1 (Fig. 3 B).

Sugar composition of LPS and lipid A

Sugar analyses of *R. salinarum* LPS showed three hexuronic acids: galacturonic acid (GalA), glucuronic acid (GlcA), and 4-*O*-methyl-galacturonic acid (4-*O*Me-GalA) in addition to the two amino sugars, GlcN and DAG. 4-*O*-Methyl-galacturonic acid, so far not described as an LPS constituent, was characterized as 4-*O*-methyl-galactitol by reduction of its carboxylic group with $NaBD_4$ and GC-MS analysis. Glucose (Glc), galactose (Gal), and L-glycero-D-manno-heptose (LD-Hep) were detected as neutral sugars (Table 1). For determination of hexuronic acids simultaneously with their respective hexoses, it was necessary to label the C6-position of the hexuronic acids with deuterium by carboxy reduction. The ratio of hexoses and hexuronic acids was calculated by comparing the different fragments containing the labeled and the unlabeled C6.

Splitting off the lipid A moiety from LPS was possible under mild conditions (1% acetic acid, 100° C, 90 min), but the yield of lipid A was only about 24% (based on LPS dry weight). The amino sugar analysis of lipid A by GC-MS showed GlcN (286.9 nmol/mg lipid A dry weight) and DAG (53.4 nmol/mg) in a ratio of 5.4:1.

Phosphate analysis

The content of phosphorous in LPS was 1.5% (based on LPS dry weight). Two phosphomonoesters, one ester-

Table 2 Comparison of the Na^+, K^+,Mg^{2+}, and Ca^{2+} content of lipopolysaccharide (LPS) and lipid A from *Rhodospirillum salinarum* strain 40. Values are given in % of LPS and lipid A dry weight

	Cation			
	Na^+	K^+	Mg^{2+}	Ca^{2+}
LPS	3.1	1.3	0.8	3.6
Lipid A	3.0	0.9	0.2	1.2

linked and one glycosidically linked, were detected by ^{31}P NMR at positions + 2.07 and + 1.23 ppm (at pH 7.29) and at position + 4.32 and + 2.50 ppm (at pH 9.86). The glycosidically linked (split with 0.1 M HC 100°C, 1 h) and the ester-linked phosphate group (+ 4.32 ppm at pH 9.86) were not substituted.

Cation analysis of LPS and lipid A by AAS

The Na^+, K^+, Mg^{2+} and Ca^{2+} contents of the *R. salinarum* LPS and lipid A, determined by atomic absorption spectroscopy (AAS), showed higher amounts of cations in the LPS fraction than in lipid A, indicating the existence of additional negative charges also in the core region (Table 2).

Laser desorption-mass spectrometric analyses (LD-MS)

For the elucidation of the backbone composition of lipid A, we analyzed the dephosphorylated and de-*O*-acylated lipid A by LD-MS. The resulting LD-mass spectrum depicted in Fig. 4 showed that we were dealing with a so-called "mixed-lipid-A-type", comprised of three distinct 1,4′-bisphosphorylated β (1→6)-linked backbone hexosaminyl-hexosamine disaccharides: (1) GlcN→GlcN (Fig. 4 A), with m/z 928 as the main fragment of its lipid A structure; (2) 2,3-diamino-2,3-dideoxy-D-Glc (DAG→DAG; Fig. 4 C), with m/z 1379; and (3) the hybrid form DAG→–GlcN (Fig. 4 B), with m/z 1154 as the main ion. The amide groups at positions 2 and 2′ of the reducing and the nonreducing sugars, respectively, were substituted in each case by a 3-OH-18 : 0. In the cases of DAG→DAG and DAG→GlcN, the additional amide groups were substituted by 3-OH-14 : 0. The peak at m/z 710 represents the

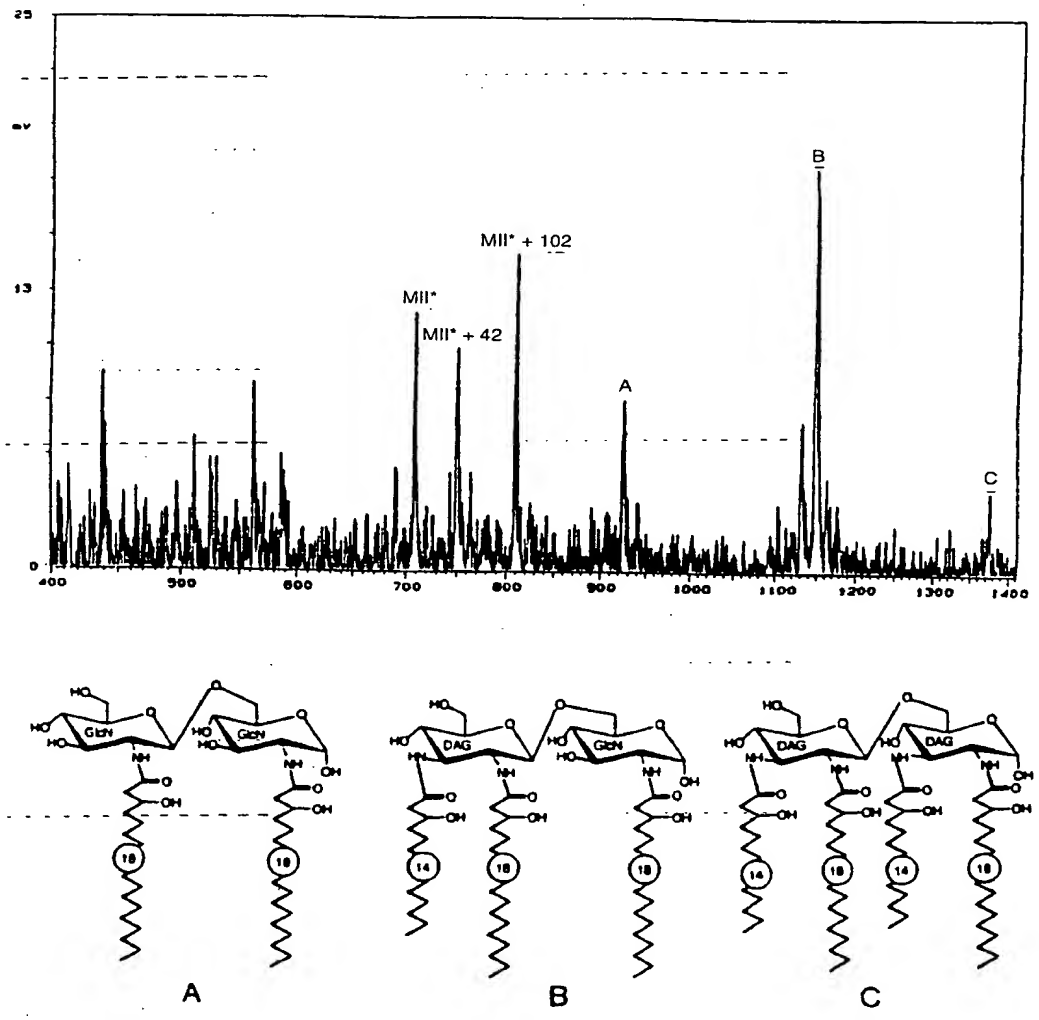


Fig. 4 Laser desorption mass spectrum of dephosphorylated, de-*O*-acylated lipid A from *Rhodospirillum salinarum* strain 40. Na^+ ions were used for ionization. *A–C* Structures of a mixed lipid A from *Rhodospirillum salinarum* strain 40. [*M*II* Amino sugar (DAG) and substituting fatty acids at the non-reducing end of structures *B* and *C*; DAG was detected only in the structures B and C]

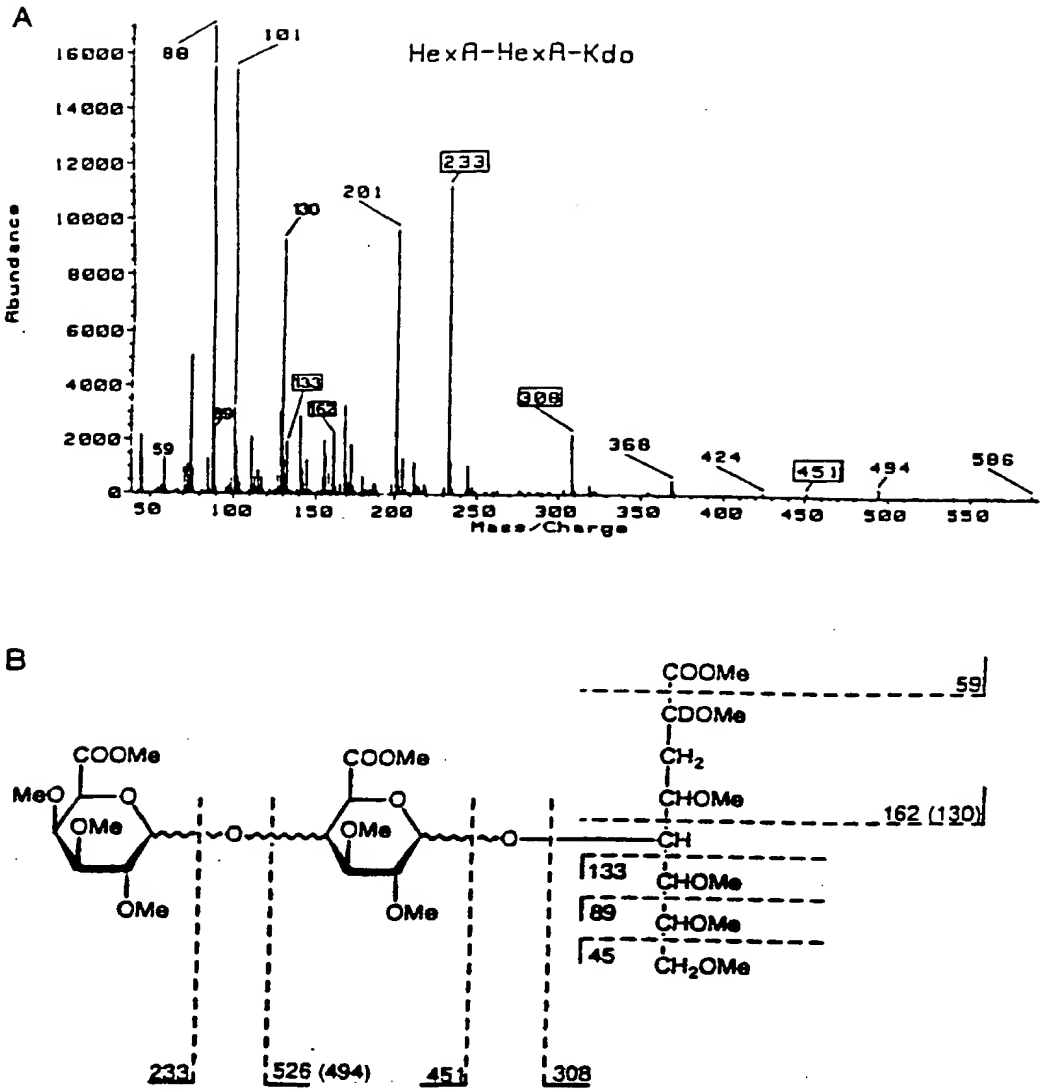


Fig. 5 A Electron impact mass spectrum and B fragmentation scheme of the $NaBD_4$-reduced and permethylated HexA-→HexA→Kdo-trisaccharide from the *Rhodospirillum salinarum* core oligosaccharide (*Me* $-CH_3$). Important primary fragments are framed

nonreducing DAG subunit (M*II), those at m/z 752 and 812 result from double-ring cleavages within the reducing sugar between the ring oxygen and C1 and C4 and C5 and between C1 and C2 and C3 and C4, respectively, leading to shifts of MII* by 42 and 102 mass units, respectively.

Analysis of a core-derived acidic trisaccharide

After reduction with $NaBD_4$ and methylation of the "degraded polysaccharide", GC-MS analysis revealed a trisaccharide HexA(1→4)HexA(1→5)Kdo_{red}. Figure 5 shows the mass spectrum (A) and the fragmentation scheme (B) of this core-derived trisaccharide from *R.salinarum* strain 40. Its EI mass spectrum showed characteristic peaks at m/z 130, 162, 201, 233, 308, 368, 451, 494, and at m/z 586. The fragment m/z 308 originated from reduced Kdo. The fragments m/z 162 and 130 (162–32) include that part of Kdo containing C1 to C4. A substitution of Kdo in this region could thus be excluded. Since the expected fragment m/z 206 comprising C1 to C5 of the reduced Kdo and its secondary fragments m/z 174 (206–32) and 142 (174–32) were not detected, it was concluded that Kdo carries a substituent at position 5. The fragments m/z 233 and m/z 201 (233–32) indicated the presence of a hexuronic acid. However, two adjacent hexuronic acids were present, judged from the fragment m/z 451. The fragment m/z 88, containing methoxylated C2 and methoxylated C3, arose from a splitting of the linkage between C1 and C2 and between C3 and C4, respectively, in a hexuronic acid unit, and gave the highest intensity in the whole spectrum. The fragment m/z 101 could be explained by the presence of two neighboring hexuronic acids in the trisaccharide-containing methoxylated C2, C3, and C4 groups in the hexuronic acids. It originated from a splitting between C1 and C2 (J series) (Kochetkov and Chizhov 1966). The J_1 ion m/z 586 included a disaccharide consisting of the reduced and methylated Kdo and a methylated hexuronic

acid containing C1 with a methyl group. This fragment was split off, and a J_2 ion m/z 526 (586–60) appeared. The loss of methanol (M = 32) created the fragment m/z 494, which was also detectable in the mass spectrum. Another J_1 ion (m/z 368) arose from the same fragmentation mechanism, but fragmentation took place in the hexuronic acid adjacent to Kdo. In this case, the J_1 ion only included the reduced and methylated Kdo and the fragment split off from the hexuronic acid. Thus, an ion m/z 308 (368–60) of the J_2 series was created.

LPS of *R. salinarum* had a lethality for mice (C57BL/10ScSN mice) of 1/10–1/100 compared with that reported for *Salmonella abortus equi* LPS (LD_{50} between 0.01 µg and 0.001 µg) (Freudenberg and Galanos 1991). *R. salinarum* LPS also induced TNFα and IL6 generation in macrophages. In these in vitro test systems (data not shown), LPS of *R. salinarum* had 10% the activity of that of *Salmonella abortus equi* LPS.

Discussion

So far, only two moderate halophilic *Rhodospirillum* species, *R. salexigens* (Drews 1981), and *R. mediosalinum* (Kompantseva and Gorlenko 1984), and one obligate halophilic species, *Rhodospirillum sodomense* (Mack et al. 1993) have been described in addition to *R. salinarum*. Nothing is known about the presence of lipopolysaccharides in the cell walls of *R. mediosalinum* and *R. sodomense*; however, in the cell-envelope fraction of *R. salexigens*, in addition to peptidoglycan and protein as the main components, a polysaccharide has been observed which is, however, not an LPS. A surface layer (S-layer) composed of a single (glyco) protein species (68 kDa) has been observed as the outermost layer by electron microscopy (Evers et al. 1984, 1986).

In *R. salinarum*, in addition to LPS, an ornithine lipid was obtained by phenol/water extraction and was separated from LPS. Ornithine lipids are membrane components from many bacterial species, including members of the former *Rhodospirillaceae* family (Imhoff et al. 1982). The two long chain fatty acids 3-OH-22:0 and 3-OH-24:0, described from the LPS fraction of *Rhodospirillum rubrum*, probably also originated from a similar ornithine lipid (Pietsch et al. 1990). Comparable ornithine lipids have been found in another bacterium of the α-1 group, *R. molischianum* (Imhoff et al. 1982), but in the LPS fraction of *R. salinarum*, no such long chain fatty acids were found. Further investigations on the ornithine lipid(s) of *R. salinarum* have not yet been done.

The Na^+, Mg^{2+}, Ca^{2+}, and K^+ contents of the LPS of *R. salinarum* were high (Table 2), as expected from the observed increased charge density in the cell envelopes of halophilic bacteria. Membranes can be stabilized by the so-called "charge screening" (Thiemann and Imhoff 1991). High contents of cations in the LPS fraction of *R. salinarum* may, therefore, be taken as an indication for the presence of highly charged molecules. The Na^+ contents of LPS and lipid A of *R. salinarum* were similar, but the K^+, Mg^{2+}, and Ca^{2+} ions were found in smaller amounts in lipid A than in LPS. From the predominance of cations in the LPS of *R. salinarum* one could assume an accumulation of negative charges in the core region. In fact, a HexA→HexA→Kdo-trisaccharide was identified by GC-MS in the "degraded polysaccharide" fraction.

The two diesters, 3-*O*-(c19:0)-14:0 and 3-*O*-(*cis*Δ^{11}-18:1)-18:0, detected in the LPS-fraction of *R. salinarum* have so far not been reported to be present in any other LPS. The two fatty acids c19:0 (*cis*-11,12-methyleneoctadecanoic acid) and *cis*Δ^{11}-18:1 are biosynthetically related and are formed in a one-step reaction by transfer of a methyl group from *S*-adenosylmethionine to 18:1 (Thiele et al. 1971). In the *R. salinarum* LPS, the hydroxy fatty acids were present in much greater amounts than the non-hydroxylated fatty acids.

3-OH-18:0 has also been detected in *Rhodopila globiformis* LPS (Pietsch et al. 1990), also a representative of the α-1 group, but generally the fatty acid spectra of the different species belonging to the α-1 group of *Proteobacteria* are rather diverse.

In the LPS of *R. salinarum*, uronic acids (GlcA and GalA) were detected in addition to 4-*O*Me-GalA, a component so far not reported in bacterial polysaccharides. In EPS ("extracellular polysaccharide") of *Rhizobium*, however, another 4-*O*-methylhexuronic acid, 4-*O*-methylglucuronic acid, has been reported (Kenne and Lindberg 1983). Due to the presence of these acidic sugars, negative charges accumulate in the LPS fraction. Comparing the LPS composition of *R. salinarum* with that of other species of the α-1 group, e.g., *R. fulvum* DSM 117, *Aquaspirillum itersonii* subsp. *nipponicum* IFO 13615, and *Aquaspirillum polymorphum* IFO 13961, uronic acids were always present in high amounts in these LPSs (Rau et al. 1993). In phototrophic bacteria and in *Rhizobia*, they may be components of the inner core region, substituting the Kdo unit directly attached to the lipid A, usually at position 4 (Mayer et al. 1990a). The HexA→HexA→Kdo trisaccharide found in the core oligosaccharide of *R. salinarum* LPS is similar to a structure previously reported to be in the inner core region of *Rhodocyclus gelatinosus* LPS αGalA(1→4)αGalA(1→4)αKdo(2→6′)-lipid A (Masoud et al. 1991). In the case of *R. salinarum*, however, Kdo was substituted at position 5 by a hexuronic acid disaccharide, whereas in *R. gelatinosus*, Kdo is substituted at position 4. A substitution of Kdo by galacturonic acid at positions 4 and 5 has been proven in the LPS of *Rhizobium leguminosarum* bv. *phaseoli* (Bhat et al. 1991). In the case of *Haemophilus influenzae*, only a single Kdo is present and this is phosphorylated at position 4, or alternatively at 5 (Helander et al. 1988).

The sequence of uronic acids in the trisaccharide of the *R. salinarum* core oligosaccharide, determined by methylation analysis, is so far unknown, but three hexuronic acids, GlcA, GalA, and 4-*O*Me-GalA, were identified (GalA:4-*O*Me-GalA:GlcA in molar ratios of 7.2:3.0:1.0). It is likely that the terminal hexuronic acid of the trisaccharide is 4-*O*Me-GalA and that GlcA is only a contamination. This must be proven, however, by methylation analysis

with C^2H_3J and also the anomeric linkages of the individual components of this trisaccharide. Perhaps Kdo and other charged groups in its direct neighborhood are important for essential functions in the outer membrane by catching distinct ions (ion trap) (Din et al. 1993).

The structural elucidation of the *R. salinarum* lipid A by laser desorption mass spectrometry revealed a mixed lipid A composed of three distinct β(1→6)-linked backbone hexosaminyl-hexosamine disaccharides: those composed of GlcN→GlcN, DAG→DAG, and DAG→GlcN. This kind of "mixed lipid A" has been hitherto investigated in detail only once with the lipid A fraction of *Campylobacter jejuni* CCUG 10936 (serotype O:2), in which the same three disaccharides GlcN→GlcN, DAG →DAG, and DAG→GlcN were detected in molar ratios of 1:1.2:6 (Moran et al. 1991). The diamino sugar (DAG) was first reported from the phosphate-free lipid A "backbone" of two phototrophic bacteria of the α-2 group of Proteobacteria: *Rhodopseudomonas viridis* and *Rhodopseudomonas palustris* (Roppel et al. 1975). The only other bacterium of the α-1 group, however, so far reported to possess a mixed-lipid-A-type is *Rhodopila globiformis*, with a lipid A containing DAG, accompanied by a small amount of GlcN (Pietsch et al. 1990). Normally, lipid A with DAG as amino sugar component is observed mainly with bacteria of the α-2 subgroup. "Mixed"-lipid-A-types with GlcN and DAG have been furthermore detected in most Chromatiaceae and Ectothiorhodospiraceae (Meissner et al. 1988; Weckesser and Mayer 1988; Zahr et al. 1992).

In laser desorption mass spectrometry, the hybrid type (DAG→GlcN), and the two homotypes with GlcN disaccharide (GlcN→GlcN) and 2,3-diamino-2,3-dideoxy-D-glucose disaccharide (DAG→DAG) were found in a ratio of 5:2:1 based on the respective fragment intensities. To determine the real ratios of the different lipid A "backbone"-types, however, it would be necessary to proceed as reported in the case of *Campylobacter jejuni* CCUG 10936 (serotype O:2) by Moran et al. (1991).

Two phosphomonoesters were recognized in the lipid A, one glycosidically linked at C1 of the reducing amino sugar and one ester-linked at C4′ of the nonreducing amino sugar. In some other species of the α-1 group of *Proteobacteria*, there are also phosphate-substituted lipid As *[Rhodospirillum molischianum, Rhodospirillum rubrum, Rhodopila globiformis, Azospirillum brasilense,* and *Azospirillum lipoferum* (Choma et al. 1987)], but other species possess phosphate-free lipopolysaccharides: *R. fulvum, A. itersonii* subsp. *nipponicum,* and *A. polymorphum* (Rau et al. 1993).

The biological actvity of the *R. salinarum* LPS was investigated by in vivo and in vitro assays. LPS showed a lethal toxicity amounting to 10^{-1}–10^{-2} of that reported for LPS of *Salmonella abortus equi*. Because of too few animals, the exact determination of the LD_{50} was not possible. The observed toxicity cannot be caused by a non-LPS contamination since C57BL/10 ScCR mice (LPS "nonresponder" mice) used as a control were unaffected. It is, however, not surprising for a bis-phosphorylated lipid A to be toxic, and the observed reduced toxicity, in comparison to the bis-phosphorylated enterobacterial lipid A, can be explained by the lower extent of acylation (only small numbers of diesters in lipid A). It has been discussed that endotoxicity may not solely be deduced from the primary chemical structure, but may depend rather on the supramolecular structure of the lipid A (Brandenburg et al. 1993; Seydel et al. 1993); this was shown to be dependent among others on the extent of acylation.

It has previously been observed with lipopolysaccharides of *Pseudomonas diminuta, Bradyrhizobium lupini, Rhizobium meliloti,* and *Rhodobacter capsulatus* that cytokine induction in macrophages parallels the in vivo lethality of D-galactosamine-treated mice (Loppnow et al. 1990). The *R. salinarum* LPS expectedly induced TNFα and IL6 in macrophages of C57BL/10 ScSN mice, and the value (about 1/10 of the activity of enterobacterial lipid A) is in accordance with the observed lethality values.

Acknowledgements The authors thank the BMFT for financial support. The technical help by E. Metz, C. Steidle and C. Warth is gratefully acknowledged. The authors thank H. Lüthje for skillful technical assistance (LD-MS). The authors thank Prof. R. Russa for helpful discussions.

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Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

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LARGE-SCALE FRACTIONATION OF S-FORM LIPOPOLYSACCHARIDE FROM *SALMONELLA ABORTUS EQUI*

CHEMICAL AND SEROLOGICAL CHARACTERIZATION OF THE FRACTIONS

CHRIS GALANOS, BINGHUA JIAO*, TETSUO KOMURO**, MARINA A. FREUDENBERG and OTTO LÜDERITZ*
Max-Planck-Institut für Immunobiologie, D-7800 Freiburg (F.R.G.)

SUMMARY

The S-form lipopolysaccharide of *Salmonella abortus equi* was separated by a newly elaborated extraction method with organic solvents into three fractions of different chain length of the O-polysaccharide they contained. The three fractions were designated long-chain (20–50 repeating units), short-chain (0–6) and R-fraction (no repeating units) according to their migration pattern in polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate. The nature of the fractions as long- and short-chain and as R-fraction was confirmed by chemical analysis. The concentration of O-specific sugars was highest in the long-chain fraction, where their molar ratio to glucosamine was *ca.* 25:1. In the short-chain fraction the ratio of O-sugars to glucosamine was 2.5:1, and in the R-fraction O-specific sugars were absent. The serological properties of the three fractions were in good agreement with their chemical composition.

INTRODUCTION

Lipopolysaccharide (LPS) represents an essential constituent of the outer membrane of the gram-negative bacterial cell. It functions as the main heat-stable antigen, the O-antigen, and is at the same time the endotoxin of these bacteria[1,2]. As such, LPS is an important factor in pathogenicity, being responsible for many pathophysiological effects occurring in gram-negative infection. LPS are constructed according to a common general principle[3]. They consist of a polysaccharide region containing the O-specific chain and the core, and a lipid, the so-called lipid A. The O-chain is built up of repeating units of oligosaccharides. In contrast to LPS from wild-type S- (smooth) form bacteria, LPS from mutant (LPS-detective) R- (rough) form bacteria lack the O-chain, thus containing only core, or fragments of it, and lipid A. Since

* Permanent address: Second Military Medical University, Shanghai, China.
** Permanent address: National Institute of Hygiene Sciences, Osaka 540, Japan.

0021-9673/88/$03.50

lipid A represents the biologically active centre of LPS, defective R-form LPS also represent highly active endotoxins[4].

LPS derived from S-form bacteria exhibit a high degree of heterogeneity, which is caused by the presence of molecules with different length of O-chains, *i.e.* with different numbers of repeating units[5–8], which may range from zero (R-form LPS) to 50. This heterogeneity becomes evident when LPS are analysed by polyacrylamide gel electrophoresis (PAGE), in the presence of sodium dodecylsulphate (SDS). In this way S-form preparations are resolved into numerous bands with a ladder-like pattern.

The migration distances of the various bands are inversely related to their molecular masses and reflect the number of repeating units present in the O-chain of the different fractions. All LPS analysed so far by SDS-PAGE have been found to contain R-form material, and therefore the biological behaviour of S-form preparations as studied so far represents mainly the combined effect of both LPS classes.

S- and R-form LPS are known to exhibit differences in their biological behaviour even though their toxic activities are very similar. One such difference is concerned with their organ distribution *in vivo*. Thus whereas the hepatic uptake of S-form LPS from the blood is effected exclusively by sinusoidal (Kupffer) cells, that of the R-form is effected both by Kupffer cells and hepatocytes, *i.e.* in contrast to S-form, R-form LPS has direct access to hepatocytes[9]. Another important difference exists in the interaction of S- and R-form LPS with high density lipoprotein *in vivo*, the result of which is that large amounts of S-form LPS accumulate in the adrenal glands whereas only insignificant amounts of R-form LPS are found in this organ[10]. S- and R-form LPS also show large differences in their ability to induce chemiluminescence in granulocytes[11], R-form LPS being very potent whereas S-form LPS are virtually inactive. Finally, S- and R-forms show distinct qualitative differences in their interactions with the complement system[1,2].

It seemed important therefore to resolve S-form LPS into fractions of S-form and R-form LPS. This would enable chemical and biological studies on genuine S-form LPS and on the R-form LPS synthesized by S-form bacteria. This paper reports the separation of S-form LPS from *Salmonella abortus equi* into fractions containing long-chain, short-chain, and R-form (no chain) LPS. The chemical compositions and serological properties of the three fractions are investigated and compared with those of the starting LPS.

EXPERIMENTAL

Lipopolysaccharides

LPS of *S. abortus equi* was isolated by the phenol–water method and subsequently purified by the phenol–chloroform–light petroleum (b.p. 40–60°C) procedure, and by ultracentrifugation[13]. Finally the LPS was electrodialysed and converted into its triethylamine salt form[13].

Sugar and fatty acid analysis

Neutral sugars were measured as their alditol acetate derivatives by gas–liquid chromatography (GLC) (Varian 1400), after hydrolysis of LPS in 0.1 *M* hydrochloric acid at 100°C for 48 h[14]. Xylose was used as internal standard. Fatty acids were

measured as methyl esters by GLC[15] in a Varian gas–liquid chromatograph (Model 3700) equipped with a flame ionization detector connected to a Hewlett-Packard integrator (Model 3380). 3-Hydroxydodecanoic and heptadecanoic acids were used as internal standards.

Colorimetric methods

Total phosphorus and glucosamine were measured according to refs. 16 and 17, respectively. 2-Keto-3-deoxy-D-manno-octonate (KDO) was determined by the thiobarbituric acid method as modified by Karkhanis *et al.*[18]. Abequose was estimated by the method of Cynkin and Ashwell[19].

Sodium dodecylsulphate-polyacrylamide gel electrophoresis

SDS-PAGE analysis of LPS and fractionated materials was performed in a discontinuous buffer system with a 5% stacking and a separating gel, according to Laemmli[7]. Gel size and thickness were 20 × 20 × 0.15 cm. A 2-μg preparation in 5 μl was loaded in each well. The current was 15 mA for stacking, and 20 mA for separating; LPS was detected by silver staining[8].

Serological assay

The antigenic properties of the three fractions obtained from *S. abortus equi* LPS were investigated by the passive haemolysis and passive haemolysis-inhibition test as described previously[20]. The three fractions were tested in relation to their ability to interact with anti-*S. abortus equi* O-antibodies and with antibodies to the various R classes (Ra–Re). The antisera used were raised in rabbits immunized with the corresponding heat-killed bacteria as described previously[20].

RESULTS

Fractionation of the lipopolysaccharide

The LPS was suspended in solvent A (chloroform–methanol–0.2 *M* hydrochloric acid, 138:127:41.5 v/v; 40 ml/g LPS) and stirred at 4°C for 15 min. After centrifugation (600 *g*, 10 min) a lower phase containing short-chain and R-fraction, and a small upper phase containing insoluble long-chain fraction were obtained. The two phases were separated, neutralized with triethylamine and evaporated. The solid materials were redissolved in water, electrodialysed, and freeze-dried.

The crude long-chain fraction was purified by repeated extraction with solvent A (two or three times).

The crude short-chain fraction, which contained R-LPS, was further fractionated by extraction with solvent B [chloroform–methanol–light petroleum–8 *M* hydrochloric acid, 12.5:4:4.5:0.2 (v/v)]. After centrifugation as above, the lower phase was enriched in R-LPS, the upper in short-chain LPS. Both fractions were isolated and then subjected to extraction with solvent C [chloroform–methanol–light petroleum–8 *M* hydrochloric acid, 10:4:2.5:0.25 (v/v)]. The procedure for isolation of the fractions was as above. In this way, pure R-fraction and short-chain fraction (still contaminated with R-LPS) were obtained.

The three fractions were designated long-chain (yield 30% based on the original LPS), short-chain (50%), and R-fraction (10%).

TABLE I

CONSTITUENTS OF *S. ABORTUS EQUI* LIPOPOLYSACCHARIDE AND THE SUBFRACTIONS

All preparations contain about three glucosamine residues (one in the core, two in lipid A).

Constituent	*Long-chain fraction*		*Short-chain fraction*		*R-fraction*		*Original lipopolysaccharide*	
	nmol/mg	*mol/GlcN*	*nmol/mg*	*mol/GlcN*	*nmol/mg*	*mol/GlcN*	*nmol/mg*	*mol/GlcN*
Sugars								
Abequose	882	8.6	335	0.8	9	0	669	3.0
Rhamnose	857	8.4	325	0.8	0	0	656	2.8
Mannose	808	7.9	307	0.7	0	0	663	2.8
Galactose	1159	11.4	700	1.6	359	0.4	1062	4.5
Glucose	193	1.9	552	1.3	400	0.5	232	1.0
Heptose	55	0.5	411	1.0	283	0.3	145	0.6
KDO	53	0.5	215	0.5	382	0.5	104	0.4
Glucosamine	102	1.0	432	1.0	810	1.0	236	1.0
*Fatty acids**								
12:0	23	0.2	130	0.3	251	0.3	51	0.2
14:0	13	0.1	102	0.2	301	0.4	21	0.1
16:0	10	0.1	75	0.2	125	0.2	20	0.1
2-OH-14:0	10	0.1	45	0.1	35	0.1	37	0.2
3-OH-14:0	95	0.9	557	1.3	1247	1.5	281	1.2
Phosphate	156	1.5	604	1.4	1054	1.3	349	1.5

* 12:0, 14:0, 16:0 are dodeca-, tetradeca- and hexadecanoic acid; 2-OH- and 3-OH- indicate 2- and 3-hydroxy.

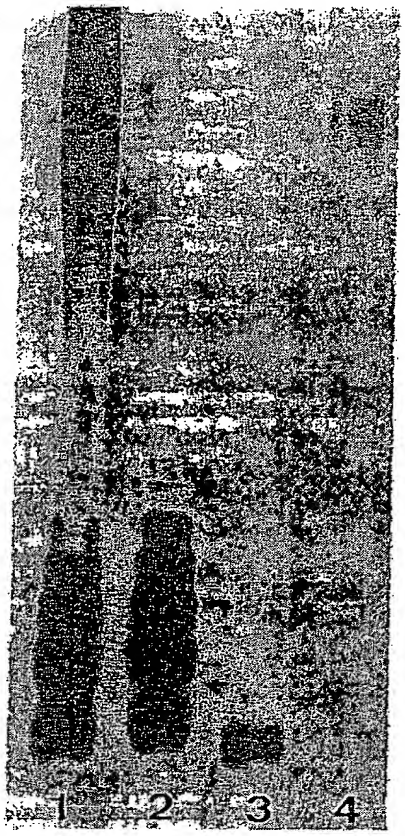

Fig. 1. SDS-PAGE of original *S. abortus equi* lipopolysaccharide and the fractions. 1 = Original LPS; 2 = short-chain fraction; 3 = R-fraction; 4 = long-chain fraction.

SDS-PAGE analysis

The electrophoretic pattern of the fractions in SDS-PAGE is shown in Fig. 1. Long-chain LPS consisted of molecules with 20–50 repeating units in the O-polysaccharide part. This value is obtained assuming that each successive band slower than the R-form band denotes an additional repeating unit in the molecule. It was free of medium-chain-length material and of R-form LPS. The short-chain fraction contained R-LPS and LPS with 1–6 repeating units. The R-fraction was devoid of O-polysaccharide.

Chemical analysis

The results of chemical analysis are shown in Table I. Sugar analysis revealed the presence of O-specific sugars in the long- and short-chain fractions and their absence in the R-fraction. Abequose, mannose and rhamnose are present in a molar ratio of *ca.* 1:1:1. The average molar ratio of each to glucosamine is 8.3:1 in the long-chain and 0.8:1 in the short-chain fraction. Since these sugars are present ex-

clusively in the O-polysaccharide, an average number of *ca.* 25 and 2.5 repeating units per molecule of long- and short-chain preparation, respectively, could be estimated, assuming three glucosamine residues (two in lipid A and 1 in the core) to be present per molecule of LPS.

All three fractions contained glucosamine, heptose and KDO in a molar ratio more or less similar to that in which they are present in the starting *S. abortus equi* LPS. Also the fatty acid content of the fractions is in good correspondence to the high or low amounts of sugars they contain. The long-chain fraction contained the lowest proportion of total fatty acids (0.15 μmol/mg) compared with the starting LPS (0.41 μmol/mg). In the short-chain fraction the concentration of fatty acids was higher than in the starting LPS (0.92 μmol/mg), and highest amounts of fatty acids were present in the R-fraction (1.96 μmol/mg).

Antigenic specificity

The serological characterization of the three fractions revealed that their antigenic specificity is in good agreement with their chemical composition. In the passive haemolysis and passive haemolysis inhibition test, the long-chain fraction reacted strongly with or inhibited anti-*S. abortus equi* O-antibodies. It showed, however, no cross-reaction with antibodies to any of the R classes (Ra to Re).

The short-chain fraction (also containing R-LPS) showed a strong cross-reaction with anti-O-antibodies and also with antibodies to Ra and Rb, but no reaction with antibodies to Rc to Re.

In contrast, the R-fraction was completely devoid of O-antigenicity. It exhibited a strong reaction with anti-Rb and a weaker reaction with anti-Ra antiserum, but no reaction with anti-Rc, -Rd and -Re.

DISCUSSION

Fractionation of the S-form LPS was based on the fact that the length of the O-chains determines the degree of lipophilicity of the molecules. Hence, the solvent mixtures that were used for sequential extraction consisted of organic solvents and increasing amounts of hydrochloric acid. These mixtures were monophasic, but small amounts of water or solutes caused separation into a lower organic phase, which contained preferentially the hydrophobic short-chain and R-LPS, and a hydrophilic upper phase with the long-chain LPS.

The success of the fractionation procedure was examined by SDS-PAGE. The only homogeneous fraction in this test seemed to be the R-LPS (yield 10% of original LPS), which was represented by one band. The short-chain fraction (50%) represented a mixture of R-LPS and LPS containing 1–6 repeating units. The number of repeating units is calculated from the numeric position of the band and by assuming that the molecular mass of bands slower than R-form material increases successively by one repeating unit. The long-chain fraction (30%), which is also heterogeneous, is devoid of R-LPS and consists of molecules containing between 20 and 50 repeating units.

The results of chemical analysis confirm the nature of the three fractions as being rough, and as containing long and short O-polysaccharide chains, respectively (Table II). Thus the R-fraction was devoid of mannose, rhamnose and abequose

TABLE II

RELATIVE MOLAR RATIOS IN THE LIPOPOLYSACCHARIDE AND THE SUBFRACTIONS OF O-SPECIFIC SUGARS AND KDO

Values based on 1 mol glucosamine. O-specific sugars are mannose, rhamnose, abequose, and part of galactose.

Fraction	*O-sugars*	*KDO*
Long-chain fraction	34	0.5
Short-chain fraction	3	0.5
R-fraction	0	0.5
Original lipopolysaccharide	11	0.4

which, together with galactose, constitute the specific sugars of the *S. abortus equi* O-antigen. The small amount of galactose found here (6.5%) was to be expected since this sugar is a constituent of the core-oligosaccharide of *Salmonella* LPS. The short- and long-chain fractions contained large amounts of O-specific sugars, the latter in a significantly higher concentration. All three fractions contained the expected core and lipid A constituents, glucose, galactose, heptose, KDO, glucosamine, phosphorus and fatty acids. Their proportions were highest in the R-fraction and lowest in the long-chain fraction.

The antigenic properties of the three fractions are in complete agreement with their chemical composition. O-antigenic reactivity was present only in the long- and short-chain fractions and was absent from the R-fraction. R-antigenic reactivity was expressed by the R-fraction and the short-chain fraction (which contains R-LPS), but not by the long-chain fraction. This indicates that the core-region in the long-chain LPS is not accessible to R antibodies. It seems therefore that cross-reactions reported frequently to occur between anti-R antibodies and S-form LPS or bacteria are due to variable amounts of R-form (and possibly short-chain LPS) present in S preparations or on the surface of the bacterial cells.

The biological properties of subfractions of S-form LPS are under study. It has been shown already that the activity of S-form LPS to induce granulocyte chemiluminescence is a property of the R-form LPS it contains, and that the long-chain fraction is completely devoid of this activity[11].

ACKNOWLEDGEMENTS

We are grateful to R. Vogt, M.-L. Gundelach and I. Minner for expert technical assistance.

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Bacterial Endotoxin

Chemical, Biological and Clinical Aspects

Edited by
J. Y. Homma, S. Kanegasaki, O. Lüderitz,
T. Shiba and O. Westphal

verlag chemie
Weinheim
Deerfield Beach, Florida
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Isolation and Purification of a Standardized Lipopolysaccharide from Salmonella abortus equi

Chris Galanos, O. Lüderitz, O. Westphal

Max-Planck-Institut für Immunbiologie, Stübeweg, D-7800 Freiburg, F.R.G.

Abstract

A standardized lipopolysaccharide designated as Novo-pyrexal has been prepared from Salmonella abortus equi. The lipopolysaccharide is free of protein, nucleic acid, and other bacterial contaminants, and it is present in the uniform sodium salt form. The physical, chemical, and biological properties of this preparation are reported here.

Introduction

It is more than 100 years ago since it was recognized that the pathogenicity of gram-negative bacteria is closely associated with surface components firmly bound in cell-wall, being released after death and lysis of the cells. For this reason these biologically active substances were named endotoxins, although their precise identity remained unknown for many years that followed. The development of procedures for the isolation and subsequent chemical analysis of the endotoxically active component in gram-negative bacteria enabled their chemical identification. Today we know that, chemically, endotoxins are lipopolysaccharides made up according to a common structural principle. They consist of the O polysaccharide, the core oligosaccharide, and the lipid A. The O polysaccharide is highly variable in its structure and composition among gram-negative bacteria and determines the serospecificity of the molecule and of the parent bacterial strain. The structure and composition of the core are less variable, being common to larger groups of bacteria.

Lipid A is a common constituent of all lipopolysaccharides exhibiting large similarities in structure and composition among gram-negative bacteria. (For reviews see 1,2,3). Lipid A is the part of the molecule responsible for toxicity and the other known biological activities of lipopolysaccharides (2).

Of the many lipopolysaccharides investigated so far, those of Salmonella have been best

characterized, and methods of isolation and chemical analysis modelled for their study have been applied successfully for the investigation of lipopolysaccharides from other genera.

Today, a high standard of lipopolysaccharide isolation and purification methodology has been achieved which allows the isolation of pure lipopolysaccharides in physicochemically defined form.

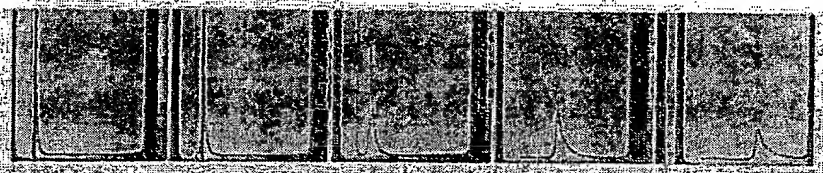
Here we report on the preparation of a standardized lipopolysaccharide derived from S.abortus equi. The preparation is free of proteins and other bacterial cell contaminants and is present in physicochemically defined form as the uniform sodium salt of the lipopolysaccharide. The preparation is also available in the form of sterile ampulled solutions that are suitable for clinical purposes and known as Novo-pyroxal*.

Results

Isolation and purification

A complete account of the methods employed has been given earlier (4). Briefly, S. abortus equi bacteria were cultivated to the late logarithmic phase as described by Schlecht (5), harvested and dried. Isolation of the lipopolysaccharide was carried out by the phenol-water procedure whereby the lipopolysaccharide was obtained in the aqueous phase (together with proteins and other bacterial contaminants). The aqueous phase was then dialyzed against distilled water, freeze-dried, and subjected to the phenol-chloroform-petroleum ether (PCP) extraction procedure whereby the lipopolysaccharide was obtained free of protein and nucleic acid. Subsequent steps in the further purification of the preparation involved ultracentrifugation, electrodialysis and re-extraction by the PCP method, as earlier described, which yielded finally the sodium salt of the S. abortus equi lipopolysaccharide.

Fig. 1. Sedimentation behaviour of the standardized S.abortus equi LPS in the triethylamine form.



Purity and physicochemical properties

The lipopolysaccharide obtained as described above was found to be free of the usual contaminants that are present in lipopolysaccharides. Thus, nucleic acids and glycogen were

*Supplied by Normal Chemie, Kurt Hermann, Reinbek b. Hamburg.

absent and the protein content measured by amino acid anaylsis was below 0.08%. Further, due to the extensive electrodialysis to which the lipopolysaccharide had been subjected, polyamines (putrescin, spermine, spermidine cadaverine etc.) which are usually present in lipopolysaccharides, ionically bound to negatively charged groups, were not detectable in the present preparation. In the analytical ultracentrifuge the lipopolysaccharide sedimented as a single sharp peak (Fig. 1), which is an additional criterium for the high purity of this material.

Table 1. Sedimentation coefficients of the Salmonella abortus equi lipopolysaccharide in different salt forms

Salt form	S value
Triethylamine	9
Ethanolamine	64
Pyridine	72
Sodium	105
Potassium	135
Putrescine	230
Calcium	partly insoluble

The S. abortus equi sodium salt lipopolysaccharide exhibits high solubility in distilled water, whereby clear solution containing up to 30 mg/ml may be obtained. When converted to other salt-forms (triethylamine, calcium etc.), the lipopolysaccharide exhibits different solubility which is chracteristic for the given salt-form, being highest in the triethylamine form and lowest in the calcium form.

The above differences in solubility are paralleled by corresponding differences in the sedimentation properties of the different salt forms in the analytical ultracentrifuge (Tab. 1). In this respect, the lipopolysaccharide of S. abortus equi behaves like other lipopolysaccharides investigated so far (6).

Chemical structure

The structure of the S. abortus equi lipopolysaccharide as elucidated during the past years is shown in Fig. 2 (7). It consists of an O polysaccharide, the core and the lipid A, a structure present in most lipopolysaccharides that have been analyzed so far. The repeating units of the O-specific chain are represented by pentasaccharides containing D-mannose, L-rhamnose and D-galactose as a linear trisaccharide, the mannose and galactose carrying branched abequose and D-glucose, respectively. Abequose and glucose constitute the sero-specific sugars in the molecule, representing the O factors 4 and 12, respectively.

The structure and composition of the core polysaccharide are as found for the genus Salmonella (8), containing 2-keto-3-deoxyoctonate (KDO), heptose, glucose, galactose and N-acetylglucosamine.

Fig. 2: Chemical structure of the S. abortus equi lipopolysaccharide

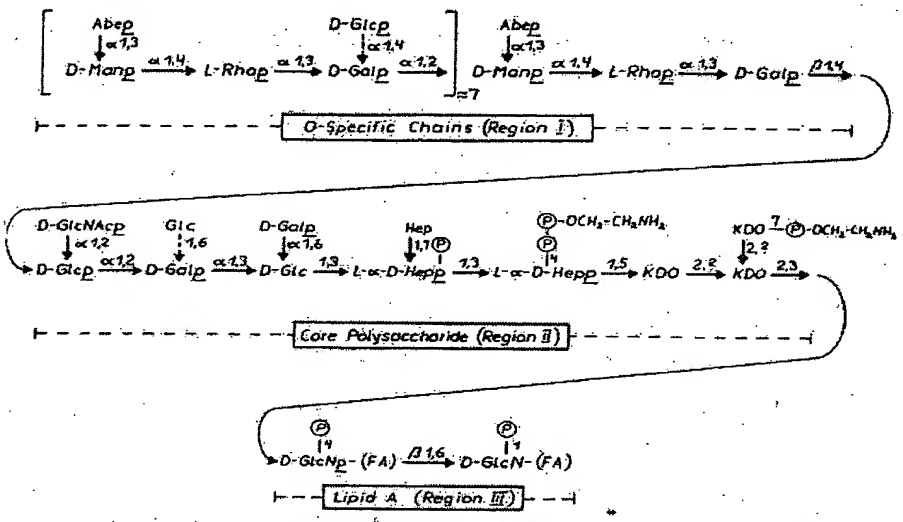


Table 2. Chemical composition of the S. abortus equi lipopolysaccharide Na-form

Sugars (unhydro)		Fatty acids (unhydro)	%	Other constituents	%
D-Abequose	13.5	Lauric (C 12)	2.2	Phosphorylresidue	5.0
L-Rhamnose	15.2	Myristic	1.8	Sodium	1.8
D-Mannose	15.7	D-3-hydroxymyristic		4-amino-L-arabinose	0.3
D-Heptose	5.0	(3-OH-C 14)	5.6	Ethanolamine	0.2
D-Galactose	17.8	Palmitic	1.1		
D-Glucose	4.0				
2-Keto-3-deoxy-D-Manno-octonic acid (KDO)	6.8				
D-Glucosamine	4.2				
Total	82.2		10.7		7.3
		Total: approx. 100 %			

The structure of the lipid A of S. abortus equi is similar to that found for most lipopolysaccharides (9). It consists of a phosphorylated D-glucosamine disaccharide in a β-1-6 linkage carrying amide linked 3-hydroxymyristic acid and ester linked lauric, myristic, palmitic and 3-hydroxymyristic acid.

Other constitutents present in the lipopolysaccharide in low concentrations are 4-amino-L-arabinose, P-ethanolamine and sodium cations.

A complete analysis of the standardized lipopolysaccharide is shown in table 2.

Figure 3: Properties of the lipopolysaccharide of S. abortus equi in different salt forms.

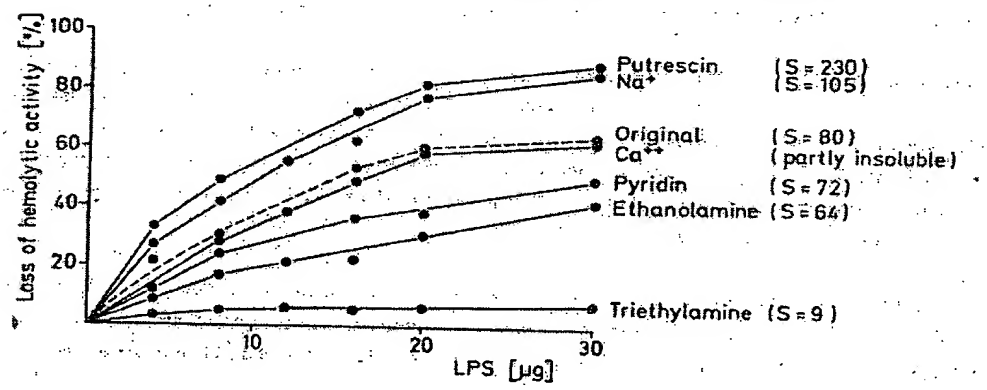
S abortus equi LPS	Sedimentation Coefficient	Solubility (Water)	Lethal Toxicity MICE RATS	Pyrogenicity (Rabbits)	Rate of clearance from the blood	Interaction with C^{1} in vivo and in vitro	Affinity for cells	Mitogenic activity
Triethylamine (TEN)	9;3	↑	↑	↑	↓	↓ (a)	↓ (b)	↑
Pyridine								
Ethanolamine								
Na								
K								
Putrescine	230							
Ca	partly insol.							

Arrows show direction of increasing activity.
(a) In the TEN form S form LPS is completely non-anticomplementary.
(b) No difference within the above range of (S) values.

Biological properties of lipopolysaccharides in uniform salt forms

The physical state of a lipopolysaccharide exerts a profound effect on its biological properties (10,11). Investigation of lipopolysaccharides in uniform salt forms which exhibit characteristic

Figure 4: Anticomplementary activity of S. abortus equi in different salt forms.



Production Manager: Dipl.-Wirt.-Ing. (FH) Myriam Nothacker

This book contains 157 figures and 110 tables.

Deutsche Bibliothek Cataloguing-in-Publication Data

Bacterial endotoxin: chem., biolog. and clin. aspects /
ed. by J. Y. Homma. – Weinheim; Deerfield Beach, Florida;
Basel: Verlag Chemie, 1984.
ISBN 3-527-26184-8 (Weinheim, Basel)
ISBN 0-89573-220-9 (Deerfield Beach, Florida)
NE: Homma, J. Y. [Hrsg.]

Printing: Betz-druck gmbh, D-6100 Darmstadt
Bookbinding: Josef Spinner, D-7583 Ottersweier
Printed in the Federal Republic of Germany

(4)

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 20, Issue of May 14, pp. 21046–21054, 2004
Printed in U.S.A.

Structure and Biological Activity of the Short-chain Lipopolysaccharide from *Bartonella henselae* ATCC 49882^T*

Received for publication, December 8, 2003, and in revised form, January 26, 2004
Published, JBC Papers in Press, February 7, 2004, DOI 10.1074/jbc.M313370200

Ulrich Zähringer‡§, Buko Lindner‡, Yuriy A. Knirel‡, Willem M. R. van den Akker¶, Rosemarie Hiestand‖, Holger Heine‡, and Christoph Dehio‖

From the ‡Research Center Borstel, Leibniz-Center for Medicine and Biosciences, 23845 Borstel, Germany, the ¶Hubrecht Laboratory, Netherlands Institute for Developmental Biology, 3584 CT Utrecht, The Netherlands, and the ‖Biozentrum of the University of Basel, Division of Molecular Microbiology, 4056 Basel, Switzerland

The facultative intracellular pathogen *Bartonella henselae* is responsible for a broad range of clinical manifestations, including the formation of vascular tumors as a result of increased proliferation and survival of colonized endothelial cells. This remarkable interaction with endotoxin-sensitive endothelial cells and the apparent lack of septic shock are considered to be due to a reduced endotoxic activity of the *B. henselae* lipopolysaccharide. Here, we show that *B. henselae* ATCC 49882^T produces a deep-rough-type lipopolysaccharide devoid of *O*-chain and report on its complete structure and Toll-like receptor-dependent biological activity. The major short-chain lipopolysaccharide was studied by chemical analyses, electrospray ionization, and matrix-assisted laser desorption/ionization mass spectrometry, as well as by NMR spectroscopy after alkaline deacylation. The carbohydrate portion of the lipopolysaccharide consists of a branched trisaccharide containing a glucose residue attached to position 5 of an α-(2→4)-linked 3-deoxy-D-*manno*-oct-2-ulosonic acid disaccharide. Lipid A is a pentaacylated β-(1′→6)-linked 2,3-diamino-2,3-dideoxyglucose disaccharide 1,4′-bisphosphate with two amide-linked residues each of 3-hydroxydodecanoic and 3-hydroxyhexadecanoic acids and one residue of either 25-hydroxyhexacosanoic or 27-hydroxyoctacosanoic acid that is *O*-linked to the acyl group at position 2′. The lipopolysaccharide studied activated Toll-like receptor 4 signaling only to a low extent (1,000–10,000-fold lower compared with that of *Salmonella enterica* sv. Friedenau) and did not activate Toll-like receptor 2. Some unusual structural features of the *B. henselae* lipopolysaccharide, including the presence of a long-chain fatty acid, which are shared by the lipopolysaccharides of other bacteria causing chronic intracellular infections (*e.g. Legionella* and *Chlamydia*), may provide the molecular basis for low endotoxic potency.

Bartonella henselae is an emerging zoonotic pathogen. In the feline reservoir host this cat flea-borne Gram-negative pathogen causes a long lasting intraerythrocytic bacteremia associated with little or no symptoms of disease (1). *B. henselae* is transmitted to humans by cat scratch or bite or the bite of an infected cat flea. Human infection results in a broad range of clinical manifestations, which often have a chronic course. Cat-scratch disease is a necrotizing inflammatory lymphadenitis typically associated with fever, which represents the most common disease manifestation in immunocompetent patients. Prolonged febrile bacteremic syndrome is a frequent disease manifestation of immunocompromized patients, which, unlike bacteremia by most other Gram-negative bacteria, has never been reported to result in septic shock. Bacillary angiomatosis and bacillary peliosis are angioproliferative lesions also found primarily in immunocompromised hosts (2).

Angioproliferation stimulated by *B. henselae* is a remarkable pathogenic process which represents a unique model to study pathogen-triggered tumor formation. Judged from the histology of bacillary angiomatosis and bacillary peliosis lesions, bacteria are in direct contact with the endothelium, probably triggering both endothelial cell proliferation and proinflammatory activation. Therefore, endothelial cells appear to represent a specific and unique target of *B. henselae*, and a detailed analysis of the bacteria-endothelial cell interactions is thus vital for understanding the pathophysiology of this emerging infection. Human umbilical vein endothelial cells have been used as an *in vitro* model to study important steps in the interaction of *B. henselae* with endothelial cells. This facultative intracellular pathogen invades endothelial cells by two different processes, either (i) by conventional endocytosis of single bacteria or small bacterial aggregates, which results in perinuclear-localizing intravacuolar bacteria, or (ii) by the invasion of large bacterial aggregates by a host cell-driven process referred to as invasome-mediated internalization (3).

B. henselae is considered to provoke angioproliferation by at least two independent mechanisms (4): directly, by triggering proliferation (5) and inhibiting apoptosis of endothelial cells (6) and indirectly, by stimulating a paracrine angiogenic loop of vascular endothelial growth factor production by infected macrophages (7).

The lipopolysaccharides (LPS)[1] of Gram-negative bacteria are known as endotoxins, which cause the prominent patho-

* This work was supported by Deutsche Forschungsgemeinschaft Grants LI-448/1-1 (to B. L. and U. Z.) and ZA 149/3-2 (to U. Z.) and by Swiss National Science Foundation Grant 31-61777.00 (to C. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed. Tel.: 49-4537-188462; Fax: 49-4537-188406; E-mail: uzaehr@fz-borstel.de.

[1] The abbreviations used are: LPS, lipopolysaccharide; COSY, correlation spectroscopy; ESI, electrospray ionization; FT-ICR, Fourier transform ion cyclotron resonance; GLC, gas-liquid chromatography; Glc, D-glucose; GlcN, D-glucosamine; GlcN3N, 2,3-diamino-2,3-dideoxyglucose; HMQC, heteronuclear multiple-quantum coherence; HPAEC, high-performance anion-exchange chromatography; IRMPD, infrared multiphoton dissociation; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; ROESY, rotating-frame nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; 12:0(3-OH), 12:1(3-OH), 16:0(3-OH), 26:0(25-OH), 28:0(27-OH), etc., 3-hydroxydodecanoic, 3-hydroxydodecenoic, 3-hydroxyhexadecanoic, 25-hydroxyhexacosanoic, 27-hydroxyoctacosanoic acids, etc.

physiological symptoms associated with sepsis and septic shock, *i.e.* fever, leukopenia, hypertension, disseminated intravascular organ failure, and multiple organ failure. The LPS from enteric bacteria, such as *Escherichia coli* and *Salmonella enterica*, are highly potent molecules with regard to their biological, *i.e.* endotoxic activities. The lipid A portion is responsible for these activities of the enterobacterial LPS (8). Endothelial cells sense the endotoxin by soluble CD14 and toll-like receptor 4 (TLR4), resulting in the nuclear factor κB (NF-κB)-dependent activation of a proinflammatory response. This phenotype is characterized by the up-regulation of the adhesion molecules ICAM-1 and E-selectin and the secretion of proinflammatory cytokines and chemokines (9).

B. henselae infection of human umbilical vein endothelial cells results in a prominant NF-κB-dependent proinflammatory activation (10, 11). However, this process is triggered by proteinacious components of the bacteria rather than by LPS (10). A mutant defective for the bacterial type IV secretion system VirB was shown to display only minimal activation of NF-κB, suggesting that bacterial effector proteins translocated by this system into endothelial cells are responsible for the proinflammatory phenotype observed during infection with wild-type *B. henselae* (11). Moreover, in contrast to wild type, the VirB-defective mutant displayed any noticeable toxic effect on endothelial cells, even at very high infection doses (11). Assuming that LPS is not affected in the VirB-defective mutant, the LPS of *B. henselae* appears to be devoid of toxicity for endothelial cells (11). This observation together with the apparent absence of septic shock in bacteremic patients indicates that the LPS of this pathogen is of low endotoxic activity. The *B. henselae* LPS should thus be interesting in regard to structure/function analysis. This LPS is composed of a major rough-type form (R-form, lacking *O*-chain), and a minor smooth-type form (S-form, containing *O*-chain) (12). However, despite the presence of a long-chain fatty acid (13), no information is available regarding the structure of *B. henselae* LPS and lipid A. Based on the limited information available, a simliar LPS is found in the closely related species *B. bacilliformis* and *B. quintana*, which represent the only other known pathogens capable of triggering angioproliferative lesions in humans. The *B. bacilliformis* LPS is also composed of a minor S-form and a major R-form, the latter being poorly immunogenic in rabbits (14). Consistently, the *B. quintana* LPS was described to be predominantly of a "deep-rough" chemotype (15) and was further shown to possess a lower endotoxic activity on whole blood cells and endothelial cells than typical endotoxins (15, 16).

In this paper, we describe elucidation of the structure of a short-chain LPS representing the major LPS species from *B. henselae* ATCC 49882^T. The unusual structural features of this novel LPS are: (i) pentaacyl lipid A, containing a GlcN3N disaccharide bisphosphate [4′-*P*-β-D-Glc*p*N3N-(1→6)-α-D-Glc*p*N3N-1→*P*] as the lipid A backbone and a long-chain fatty acid, namely 26:0(25-OH) or 28:0(27-OH), and (ii) a small and unique inner core composed of an α-(2→4)-linked 3-deoxy-d-*manno*-oct-2-ulosonic acid (Kdo) disaccharide with one glucose residue attached. Moreover, we demonstrated that this LPS has a low endotoxic potential as measured by TLR4 signaling and, in contrast to LPS from some other pathogens, does not activate TLR2-signaling to any considerable extend. The structure and biological activity of the *B. henselae* ATCC 49882^T short-chain LPS displays interesting parallels with LPS of rhizobacteria, which are phylogenetically related intracellular plant symbionts, as well as with some human pathogens poorly related to *B. hensleae* (*e.g. Legionella* and *Chlamydia*), which, however, share the intracellular life style and the typical chronic course of infection.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Cultivation—*B. henselae* ATCC 49882^T isolated from the blood of an human immunodeficiency virus-positive febrile patient was obtained from the Collection de l'Institut Pasteur (CIP), Paris, France. *B. henselae* IBS 113 isolated from the blood of a bacteremic cat was kindly provided by Dr. Y. Piemont, Hopital Louis Pasteur, University of Strasbourg, Strasbourg, France. *B. henselae* ATCC 49882^T was grown for 3 days on Columbia agar containing 5% defibrinated sheep blood at 35 °C in a humidified atmosphere.

Small Scale Isolation of LPS for SDS-PAGE—Bacterial LPS was isolated from proteinase K-treated whole bacteria, separated by Tricine-SDS-PAGE and stained by oxidative silver staining as previously described for *Bordetella* spp. (17).

Preparative Isolation of LPS—Bacteria were harvested from 500 plates in phosphate-buffered saline and washed twice in distilled water, followed each time by centrifugation for 30 min at 18,000 × *g*. The bacterial pellet was finally suspended in a small volume of aqueous 0.5% phenol. Dried bacteria (3.1 g) were washed successively with ethanol (300 ml), acetone (300 ml), and diethyl ether (300 ml) and dried in air overnight. The pellet was suspended in water (250 ml), treated first with RNase and DNase (2 mg each) at stirring over night at room temperature, then with proteinase K (2 mg) for 24 h at 20 °C, dialyzed extensively (4 days) against distilled water, and centrifuged. The precipitate was washed with acetone, suspended in water, and lyophilized (final yield of washed bacteria: 1.63 g).

Attempts to isolate LPS by direct extraction of the digested dried bacterial cells described above using the phenol-water (P/W) (18) or phenol/chloroform/petrol ether procedures (19) were not satisfactory. After extensive enzyme digestions using proteinase K in the presence of SDS, mercaptoethanol, and lysozyme, sufficient quantities of *B. henselae* LPS for analytical studies could be extracted by the phenol/chloroform/petrol ether (2:5:8, v/v/v) procedure (19). To remove non-LPS lipids, prior to extraction cells were washed three times with a 1:1 (v/v) chloroform/methanol mixture, centrifuged, and dried in a stream of nitrogen. To remove residual protein from this crude LPS perparation, the protocol of Hirschfeld *et al.* (20) was used. An aliquot of the extract containing 5 mg of LPS gave 2.9 mg of purified LPS, which showed no banding pattern in SDS-PAGE and Coomassie Brilliant Blue staining.

O-Deacylation of LPS—LPS (14 mg) was dried over P_2O_5 and treated with anhydrous hydrazine (0.5 ml) at 37 °C for 35 min at ultrasonication. Acetone (5 ml) was added in the cold and the precipitate was separated by centrifugation, washed twice with acetone, dissolved in water (3 ml), and lyophilized. The product was treated with anhydrous hydrazine at 37 °C for 1.5 h and treated as above to give O-deacylated LPS (11 mg).

Alkaline Degradation of LPS—*O*-Deacylated LPS (11 mg) was heated with 4 M KOH (1 ml) at 120 °C for 23.5 h, and the reaction mixture was diluted with water (5 ml), neutralized at 0 °C with 2 M HCl (2 ml), and extracted with chloroform (2 × 4 ml). After separation of phases the precipitate was washed with water (2 × 2 ml), the combined water phase and washings were lyophilized. The product was desalted by gel chromatography on Sephadex G-50 in pyridinium acetate buffer, pH 4.5 (4 ml of pyridine and 10 ml of HOAc in 1 liter of water), and fractionated by HPAEC using a Dionex system with pulsed amperometric detection on an analytical CarboPac PA1 column (250 × 4.6 mm) in a linear gradient of 0.15 → 0.5 M NaOAc in 0.1 M NaOH for 70 min at 1 ml/min. 1-ml fractions were collected and analyzed by HPAEC using the same gradient for 30 min at 1 ml/min. Four products with retention times 19.25, 21.35, 24.75 (major, 300 μg), and 28.10 min in the analytical run were isolated and desalted on a column (40 × 2.5 cm) of Sephadex G-50 in pyridinium acetate buffer, pH 4.5.

Mild Acid Degradation of LPS—LPS (0.4 mg) was hydrolyzed with 0.1 M sodium acetate buffer, pH 4.4, at 100 °C for 2 h, and the supernatant was deionized with an IR-120 (H^+-form) cation-exchange resin and analyzed by HPAEC in a linear gradient of 0.01 → 0.08 M NaOAc in 0.1 M NaOH for 30 min at 1 ml/min, which showed the presence of two compounds with retention times of 23.38 and 24.55 min.

Composition Analyses—For analysis of Kdo, LPS was methanolyzed with 0.5 M HCl in methanol at 85 °C for 45 min. After removal of the solvent, the products were peracetylated with Ac_2O in pyridine (1:1.5, v/v, 85 °C, 20 min). For analysis of Glc and GlcN3N, LPS was methanolyzed with 2 M HCl in methanol at 85 °C for 16 h, then hydrolyzed with 4 M CF_3CO_2H at 100 °C for 2 h, conventionally borohydride-reduced and peracetylated (21). For fatty acid analysis, LPS was methanolyzed with 2 M HCl in methanol at 85 °C for 20 h, and sugars were trimethylsilylated with *N,O*-bis(trimethylsilyl)trifluoroacetamide (22). The sugar and fatty acid derivatives were analyzed by GLC on a

Hewlett-Packard HP 5890 Series II chromatograph, equipped with a 30-m fused silica SPB-5 column (Supelco) using a temperature gradient of 150 °C (3 min) → 320 °C at 5°/min, and GLC-MS on a Hewlett-Packard HP 5989A instrument equipped with a 30-m HP-5MS column (Hewlett-Packard) under the same chromatographic conditions as in GLC.

Methylation Analysis—*O*-Deacylated LPS was dephosphorylated with aqueous 48% hydrofluoric acid (4 °C, 16 h), methylated with CH_3I in dimethyl sulfoxide in the presence of solid NaOH (23), and hydrolyzed with 2 M CF_3CO_2H (100 °C, 2 h). The partially methylated monosaccharides were borohydride-reduced, and Kdo was converted into the methyl ester by evaporation with 0.5 M HCl in methanol, peracetylated, borohydride reduced, peracetylated, and analyzed by GLC and GLC-MS as described above.

Mass Spectrometry—MALDI-TOF MS was performed on a Bruker-Reflex II instrument (Bruker-Franzen Analytik, Bremen, Germany) in linear configuration in the negative mode at an acceleration voltage of 20 kV and delayed ion extraction. Samples were dissolved in chloroform at a concentration of 5 μg/μl, and 2 μl of solution was mixed with 2 μl of 0.5 M 2,4,6-trihydroxyacetophenone (Aldrich) in methanol as matrix solution. 1-μl aliquots were deposited on a metallic sample holder and analyzed immediately after drying in a stream of air.

ESI FT-ICR MS/MS was performed using a Fourier transform ion cyclotron resonance mass analyzer (ApexII, Bruker Daltonics) equipped with a 7-tesla actively shielded magnet and an Apollo electrospray ion source. Samples were dissolved in a 30:30:0.01 (v/v/v) mixture of 2-propanol, water, and triethylamine at a concentration of ~20 ng/μl and sprayed with a flow rate of 2 μl/min. For IRMPD MS/MS, a 40-watt CO_2 laser (Synrad Inc., Mukilteo, WA) operating at λ = 10.6 μm was used. Laser power was set to 50% of maximal value. Duration of irradiation was controlled via the XMASS software (Bruker Daltonics) for optimal fragmentation (typically 50 ms).

NMR Spectroscopy—Prior to the measurements, the samples were lyophilized twice from ^2H_2O. The ^1H and ^{31}P NMR spectra were recorded with a Bruker DRX-600 spectrometer (Bruker, Rheinstetten, Germany) at 600 and 243 MHz, respectively, at 27 °C in 99.96% ^2H_2O. Chemical shifts were referenced to internal sodium 3-trimethylsilylpropanoate-d_4 (δ_H 0) and external aqueous 85% H_3PO_4 (δ_P 0). Bruker software XWINNMR 2.5 was used to acquire and process the data. A mixing time of 100 and 200 ms was used in two-dimensional TOCSY and ROESY experiments, respectively.

Activation of HEK/HEK-CD14 Cells—For transient transfection, HEK293 cells were plated at a density of $5 \cdot 10^4$/ml in 96-well plates in Dulbecocc's modified Eagle's medium without G418. The following day, cells were transfected using Polyfect (Qiagen) according to the manufacturer's protocol. Expression plasmid containing human CD14 was a kind gift of Dr. D. T. Golenbock, Worcester, MA, and the FLAG-tagged versions of human TLR2 and human TLR4 were a kind gift from P. Nelson, Seattle, WA and subcloned into pREP9 (Invitrogen). The human MD-2 expression plasmid was a kind gift from K. Miyake, Tokyo, Japan. TLR2 and TLR4 plasmids were used at 200 ng/transfection, CD14 and MD-2 plasmids were used at 25 ng/transfection. The total DNA content was kept constant at 250 ng/transfection using pCDNA3 (Invitrogen). After 24 h of transfection, cells were washed and stimulated with LPS for another 18 h. Finally, supernatants were collected and the interleukin-8 content was quantified using a commercial enzyme-linked immunosorbent assay (BIOSOURCE).

Bacterial lipopeptide ($Pam_3CysSK4$) was obtained from EMC microcollections, Tübingen, Germany, and LPS from *S. enterica* sv. Friedenau was kindly provided by H. Brade, Borstel, Germany.

RESULTS

Purification of B. henselae LPS and Analysis by SDS-PAGE—Crude LPS extracts from *B. henselae* isolated following proteinase K treatment of whole cells was separated by SDS-PAGE and visualized by silver staining. Consistent with a recent study examining the LPS composition of several *B. henselae* strains (12), we observed for strains ATCC 49882^T (Fig. 1*A*, *lane 1*) and IBS 113 (*lane 2*) the presence of at least two LPS species of different electrophoretic mobility, an R-form of about 3 kDa as the major constituent and at least one S-form of >20 kDa as a minor constituent. LPS was then isolated in preparative scale from cells of *B. henselae* ATCC 49882^T by phenol/chloroform/petrol ether extraction (19) following extensive digestion of bacterial cells with proteolytic enzymes. Extraction with chloroform/methanol was necessary to remove non-LPS lipids. When this purified LPS was tested, no high molecular mass S-form LPS was observed (Fig. 1*B*, *lane 1*), indicating selective enrichment of the R-form during phenol/chloroform/petrol ether extraction. The mobility of the R-form LPS was in the range between that of *E. coli* deep-rough mutant strain F515 (chemotype Re, containing two Kdo residues as the core oligosaccharide; Fig. 1*B*, *lane 2*) and *S. enterica* sv. Minnesota rough mutant R7 (chemotype Rd_1, containing two Kdo and two heptose residues; Fig. 1*B*, *lane 3*). Therefore, *B. henselae* ATCC 49882^T produces a short-chain R-form LPS with a core ranged in size between di- and tetrasaccharide.

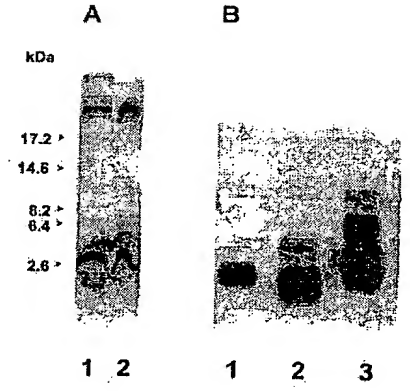


FIG. 1. **Silver-stained SDS-PAGE (20%).** *A*, small scale isolation of LPS from *B. henselae* ATCC 49882^T (*lane 1*) and strain IBS 113 (*lane 2*). *B*, purified LPS from *B. henselae* ATCC 49882^T (*lane 1*), *E. coli* mutant F515 (chemotype Re, *lane 2*), and *S. enterica* sv. Minnesota mutant R7 (chemotype Rd_1, *lane 3*).

Chemical and Mass Spectrometric Characteriztaion of LPS—Sugar analysis of the purified LPS showed the presence of Glc, Kdo, and GlcN3N, but no GlcN. Most likely, Glc and Kdo are components of the core oligosaccharide portion of LPS, whereas GlcN3N has been previously identified in the lipid A backbone of several bacteria (24). Fatty acid analysis revealed 12:0(3-OH), 16:0(3-OH), 26:0(25-OH), and 28:0(27-OH) as the major constituents, together with 12:1(3-OH), 14:0(3-OH), 18:0(3-OH) as minor constituents, and a negligible amount of 18:1(3-OH).

Methylation analysis of the *O*-deacylated dephosphorylated LPS, including carboxyl reduction of Kdo, demonstrated 4,5-disubstituted Kdo (Kdo^I), terminal Glc, and terminal Kdo (Kdo^{II}). These data indicated that the LPS core is a branched trisaccharide composed of one Glc and two Kdo residues. A minor amount of a 5-substituted Kdo residue was also detected, which, most likely, resulted from a partial elimination of the terminal Kdo residue in the course of dephosphorylation of LPS under acidic conditions (25).

The charge-deconvoluted negative ion ESI FT-ICR mass spectrum of LPS (Fig. 2) showed two major molecular ion peaks at *m/z* 2399.44 (M^I) and 2427.45 (M^{II}) and a minor peak at *m/z* 2455.50. Taking into account the composition of LPS, the major peaks could be assigned to LPS species containing a $GlcKdo_2$ trisaccharide and a bisphosphoryl di-GlcN3N lipid A backbone acylated with two residues each of 12:0(3-OH) and 16:0(3-OH) and one residue of either 26:0(25-OH) or 28:0(27-OH). The minor compound may differ in the replacement of one 12:0(3-OH) or 16:0(3-OH) residue with a residue of 14:0(3-OH) or 18:0(3-OH), respectively. Furthermore, the spectrum exhibited minor peaks representing compounds missing one phosphate group (*m/z* 2319.47, 2347.50, and 2365.51) or those containing one unsaturated fatty acid (*m/z* 2397.43, 2425.45, and 2453.50). Unlabelled peaks in Fig. 2 represent sodium adduct ions.

Positive ion ESI FT-ICR IRMPD MS/MS of triethylammo-

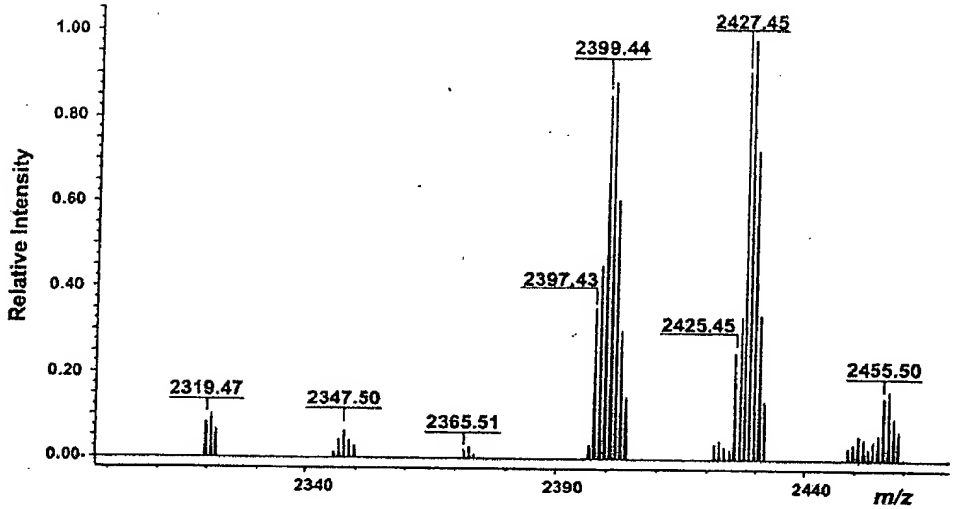


FIG. 2. **Charge-deconvoluted negative ion FT-ICR ESI mass spectrum of LPS of *B. henselae* ATCC 49882^T.**

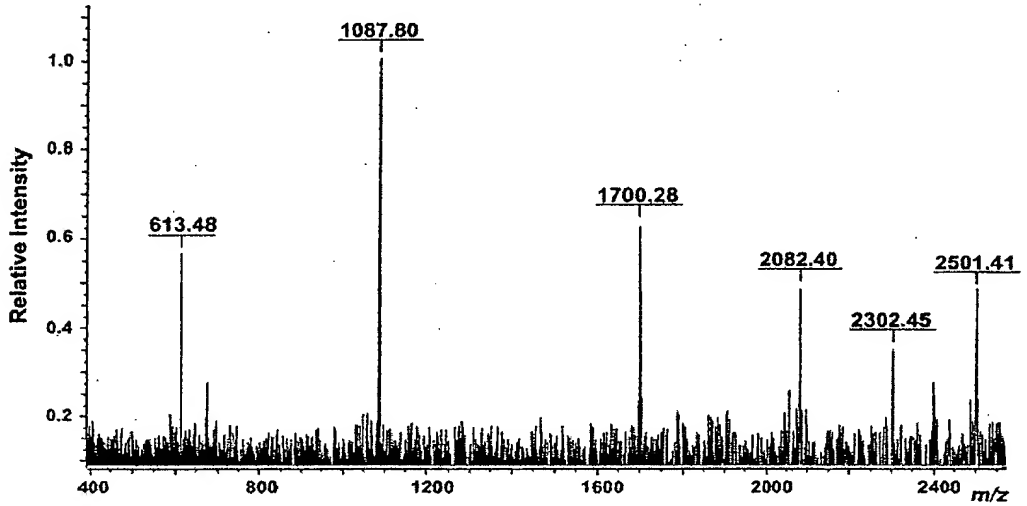


FIG. 3. **Positive ion ESI FT-ICR IRMPD MS/MS of triethylammonium salt of LPS of *B. henselae* ATCC 49882^T.** The molecular ion at *m/z* 2501.41 was used as the parent ion.

nium salt of LPS was performed using the molecular ion at *m/z* at 2501.41 (M^I-salt) as the parent ion (Fig. 3). The spectrum showed ion peaks due to subsequent loss of triethylammonium phosphate (*m/z* 2302.45) and Kdo (*m/z* 2082.40) or the $GlcKdo_2$ trisaccharide (*m/z* 1700.28, lipid A ion). The monophosphoryl lipid A ion was further cleaved to give β- and γ-fragment ions (according to the nomenclature of Domon and Costello) at *m/z* 1087.80 and an ion from the reducing end at *m/z* 613.48 (711.48, *P*), respectively. This fragmentation pattern showed that each GlN3N residue in lipid A bears one 12:0(3-OH), one 16:0(3-OH), and one phosphate group and that in M^I the 26:0(25-OH) residue is attached to the non-reducing GlcN3N residue ($GlcN3N^{II}$).

Alkaline Degradation of LPS and Structure of the Carbohydrate Core-Lipid A Backbone—LPS was *O*-deacylated with anhydrous hydrazine. As expected, the negative ion ESI FT-ICR mass spectrum of the *O*-deacylated LPS showed ion peaks at *m/z* 2005.04 (major) and 2033.08 (minor) for LPS species with four *N*-linked fatty acids. Further *N*-deacylation of the *O*-deacylated LPS resulted in a mixture of four oligosaccharide bisphosphates, which were separated by HPAEC. In negative ion ESI FT-ICR MS/MS, the major compound (*1*) with the HPAEC retention time 24.75 min showed a molecular ion peak at *m/z* 1099.28 and thus has the full core trisaccharide. The other compounds have a truncated core lacking either Kdo^{II} or Glc or both Kdo^{II} and Glc (molecular ions at *m/z* 879.22, 937.22, and 717.17, respectively) (data not shown). The content of the Kdo^{II}-lacking product with the retention time 19.25 min was twice as low as that of the major compound, whereas the two Glc-lacking products were present in negligible amounts. Since no compound with a truncated core was detected in MS studies on the whole LPS, the minor compounds resulted from partial cleavage of the glycosidic linkages under strong alkaline conditions.

Compound 1 was studied by high-field NMR spectroscopy. The ^1H NMR spectrum of compound 1 (Fig. 4) showed signals for three anomeric protons at δ 4.32, 5.22, and 5.26, protons at nitrogen-bearing carbons (H-2 and H-3) of two GlcN3N residues at δ 2.48, 2.55, 2.61, and 2.82, and methylene groups of two Kdo residues at δ 1.72, 1.92 (both H-3ax), 2.02, and 2.05 (both H-3eq). The spectrum was completely assigned using two-dimensional $^1H,^1H$ COSY, TOCSY, and $^1H,^{13}C$ HMQC ex-

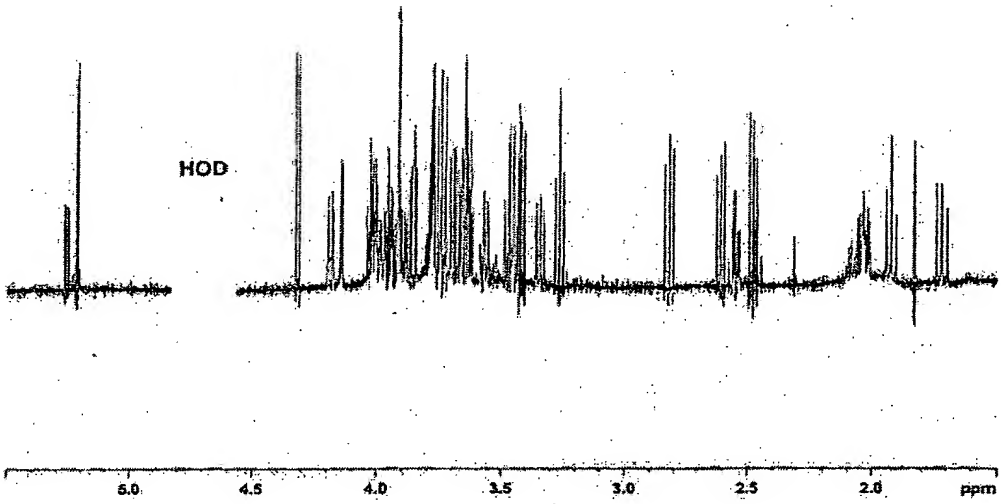


FIG. 4. **^{1}H NMR spectrum of the compound 1 isolated from LPS of *B. henselae* ATCC 49882^T by stepwise *O*- and *N*-deacylation.** For signal assignment, see Table I.

TABLE I
^{1}H NMR chemical shifts of the compound 1 from LPS of B. henselae ATCC 49882^T

Sugar residue	H-1H-3ax	H-2H-3eq	H-3H-4	H-4H-5	H-5H-6	H-6aH-7	H-6bH-8a	H-8b
α-Glc*p*-(1→	5.22	3.42	3.74	3.46	4.01	3.78	3.78	
α-Kdo*p*^{II}-(2→	1.72	2.05	3.90	3.90	3.64	3.96	3.65	3.78
→4,5)-α-Kdo*p*^{I}-(2→	1.92	2.02	4.02	4.14	3.68	3.94	3.69	3.85
→6)-β-Glc*p*N3N4*P*^{II}-(1→	4.32	2.48	2.61	3.45	3.57	3.34	3.74	
→6)-α-Glc*p*N3N^{I}-1-*P*	5.26	2.55	2.82	3.26	3.99	3.64	4.18	

TABLE II
^{13}C NMR chemical shifts of the compound 1 from LPS of B. henselae ATCC 49882^T

Chemical shifts were determined from the two-dimensional ^{1}H,^{13}C HMQC spectrum.

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8
α-Glc*p*-(1→	100.8	73.5	73.9	70.5	72.9	61.4		
α-Kdo*p*^{II}-(2→			36.1	68.0[a]	68.0[a]	73.6[a]	70.4	64.5
→4,5)-α-Kdo*p*^{I}-(2→			36.0	73.1	75.1	73.3	72.0	64.0
→6)-β-Glc*p*N3N4*P*^{II}-(1→	105.2	57.5	59.6	75.3	77.5	64.7		
→6)-α-Glc*p*N3N^{I}-1-*P*	95.7	56.7	55.0	71.5	72.8	70.8		

[a] Superposition of two non-resolved signals.

periments, and spin systems of one Glc, two GlcN3N, and two Kdo residues were identified. The ^{1}H and ^{13}C NMR chemical shifts (Tables I and II) and typical coupling constant values indicated that all sugar residues are in the pyranose form. A large $J_{1,2}$ coupling constant of 8.0 Hz for the H-1 signal at δ 4.32 showed that one of the GlcN3N residues (GlcN3N^{II}) is β-linked, whereas Glc and GlcN3N^{I} are α-linked ($J_{1,2}$ 3.5 and 3.3 Hz for the H-1 signals at δ 5.22 and 5.26, respectively). The H-1 signal of α-GlcN3N^{I} was additionally split due to coupling to phosphorus ($J_{1,P}$ 8.6 Hz). The α-configuration of both Kdo residues followed from the characteristic ^{1}H and ^{13}C NMR chemical shifts, which were similar to those of α-Kdo*p* but different from the values of β-Kdo*p* (26).

A two-dimensional ROESY experiment revealed correlations of the anomeric proton of GlcN3N^{II} with the protons at the linkage carbon H-6a,6b of GlcN3N^{I} at δ 4.32/3.64 (strong) and δ 4.32/4.18 (weak), thus indicating the β1→6-linkage between the monosaccharides in the lipid A backbone. As expected, no interresidue cross-peak was observed for H-1 of GlcN3N^{I} at δ 5.26. The anomeric proton of Glc showed correlations with H-5 and H-7 of Kdo^{I} at δ 5.22/4.14 (strong) and 5.22/3.94 (weak), respectively, which are typical of the α1→5-linkage between these sugar residues (27). Finally, a strong correlation between H-3eq of Kdo^{I} and H-6 of Kdo^{II} at δ 2.02/3.64 proved that Kdo^{I} and Kdo^{II} are α-(2→4)-interlinked (27, 28).

The glycosylation pattern was further confirmed by the ^{13}C NMR chemical shift data of the linkage carbons (Table II), whose signals typically shifted down-field compared with their positions in the corresponding non-substituted monosaccharides. The displacements were relatively large when the substituent is an aldopyranose (7–8 ppm for C-5 of Kdo^{I} and C-6 of GlcN3N^{I} caused by glycosylation with Glc and GlcN3N^{II}, respectively) and smaller in case of a ketopyranose substituent (~5 and ~2 ppm for C-4 of Kdo^{I} and C-6 of GlcN3N^{II} glycosylated with Kdo^{II} and Kdo^{I}, respectively).

The ^{31}P NMR spectrum of compound 1 contained signals for two phosphate groups at δ 4.72 and 4.94. A ^{1}H,^{31}P HMQC experiment showed correlations of the former with H-1 of GlcN3N^{I} at δ_P/δ_H 4.72/5.26 and the latter with H-4 of GlcN3N^{II} at δ_P/δ_H 4.94/3.45. Therefore, the lipid A disaccharide backbone is bisphosphorylated at positions 1 and 4′, and the compound 1 has the structure shown in Fig. 5.

Mild Acid Degradation and Full Structure of LPS—Hydrolysis of LPS at pH 4.4 cleaved the Kdo linkages, including the linkage between Kdo^{I} and lipid A. As expected, analysis of the released carbohydrate portion by HPAEC, ESI FT-ICR MS/MS, and ^{1}H NMR spectroscopy (data not shown) indicated the presence of two compounds, Kdo and a Glc → Kdo disaccharide. MALDI-TOF mass spectrum of lipid A showed the presence of two major ion peaks at *m/z* 1798.63 (M_{LA}^{I}) and 1826.64 (M_{LA}^{II})

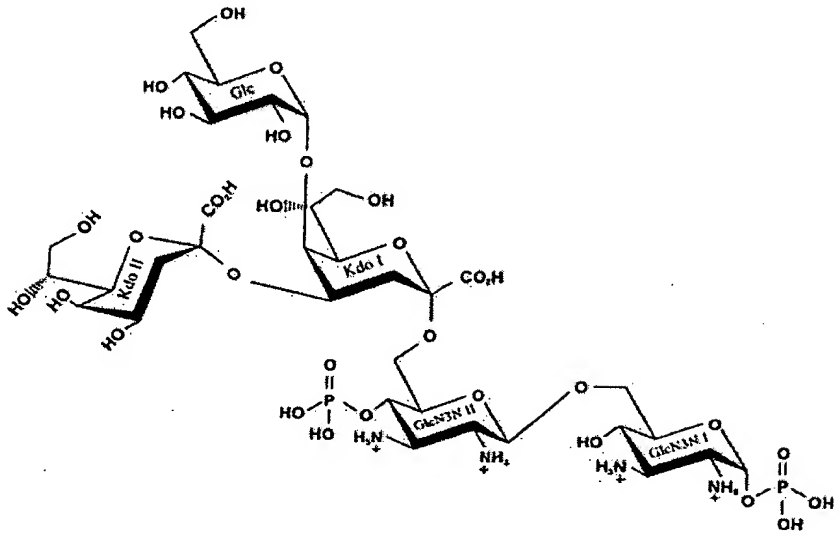


FIG. 5. **Structure of the compound 1 isolated from LPS of *B. henselae* ATCC 49882^T by stepwise *O*- and *N*-deacylation.**

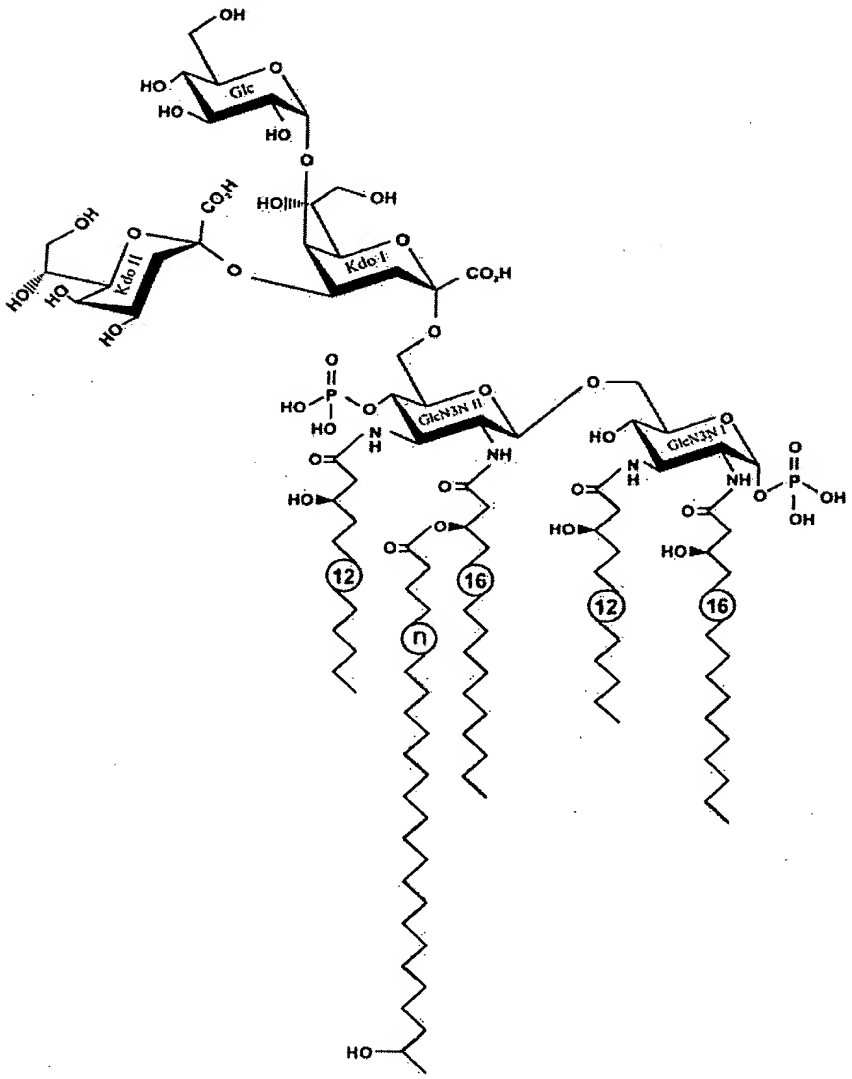


FIG. 6. **Proposed structure of two major LPS species of *B. henselae* ATCC 49882^T.** The position of 12:0(3-OH) and 16:0(3-OH) within each GlcN3N residue could be interchanged; 26:0(25-OH) and 28:0(27-OH) (n = 26 and 28, respectively) could be attached to either 16:0(3-OH), as shown, or 12:0(3-OH) on GlcN3N^{II}. Minor LPS species may differ in the replacement of 12:0(3-OH) with 12:1(3-OH) or 14:0(3-OH) and/or 16:0(3-OH) with 18:0(3-OH) or 18:1(3-OH).

in similar amounts. They corresponded to bisphosphorylpentaacyl lipid A species containing two residues each of 12:0(3-OH) and 16:0(3-OH) and one residue of 26:0(25-OH) or 28:0(27-OH), respectively. These data were in full agreement with the data obtained on the whole LPS. Minor ion peaks were also present, which could be assigned to (i) the corresponding tet-

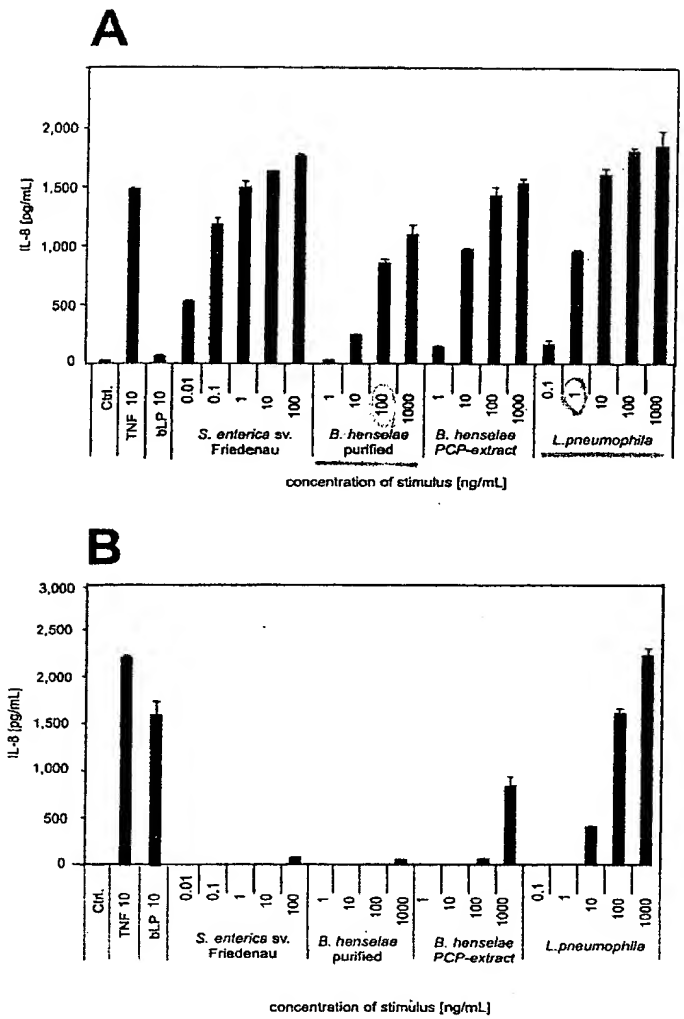


FIG. 7. **Purified LPS from *B. henselae* ATCC 49882^T activates HEK293 cells through TLR-4/MD-2.** HEK293 cells were transiently transfected with CD14 and either TLR4/MD-2 (*A*) or TLR2 (*B*) as described under "Experimental Procedures." After 24 h, cells were stimulated with indicated ligands for 18 h. Interleukin-8 content of the supernatant was analyzed by enzyme-linked immunosorbent assay. One representative experiment out of three is shown.

raacyl species lacking 26:0(25-OH) and 28:0(27-OH) (M_{LA}^{I} − 394 or M_{LA}^{II} − 422), (ii) the species with longer-chain fatty acids (M_{LA} + 28), *e.g.* those containing 14:0(3-OH) or 18:0(3-OH) instead of 12:0(3-OH) or 16:0(3-OH), respectively, and (iii) monophosphoryl species (M_{LA} − 80). No tetraacyl and no monophosphoryl lipid A species could be detected in studies of the whole LPS, and, hence, they were produced during mild acid degradation of LPS.

These data together showed that the short-chain LPS of *B. henselae* ATCC 49882^T has the structure shown in Fig. 6.

TLR2 and TLR4/MD-2-dependent Activity in HEK293 Cells—Next, we investigated which receptors are involved in the activation of cells by LPS from *B. henselae* and *Legionella pneumophila* having a similar lipid A structure (32). HEK293 cells were transiently transfected with CD14 and either TLR2 or TLR4/MD-2 and stimulated with various LPS preparations. As shown in Fig. 7, the LPS preparations from *S. enterica* sv. Friedenau, *L. pneumophila*, and *B. henselae* all showed TLR4 activity, although to a different extent. In comparison to the standard LPS preparation from *S. enterica* sv. Friedenau, *B. henselae* LPS appears to be at least 1,000-fold less active with respect to TLR4 activity. *L. pneumophila* LPS expressed a slightly higher TLR4 activity, still being 10–100-fold less than that of *S. enterica* sv. Friedenau LPS (Fig. 7*A*).

In contrast to TLR4, TLR2 activity was stimulated only by the crude LPS extracts from *L. pneumophila* and *B. henselae* but neither by the purified *B. henselae* LPS nor by that of *S. enterica* sv. Friedenau (Fig. 7*B*). The *L. pneumophila* LPS preparation showed strong TLR2 activity already at 10 ng/ml, which reached at 100 ng/ml a level comparable with the standard bacterial lipopeptide (Pam_3CysSK_4) preparation serving as positive control. The absence of CD14 in TLR2 as well as TLR4/MD-2 transfected cells reduced the activity of all tested LPS preparations but did not alter the observed differences in their activity (data not shown).

DISCUSSION

In this study, we have determined the molecular structure and biological, *i.e.* endotoxic activity of the major short-chain LPS form *B. henselae* ATCC 49882^T. This represents the first detailed LPS structure analysis of a member of the expanding genus *Bartonella*, which currently comprises 19 species of facultative intracellular pathogens, including eight species associated with human diseases (4).

The lipid A backbone of *B. henselae* ATCC 49882^T is composed of a bisphosphorylated GlcN3N disaccharide shared with only a few bacteria, including *Pseudomonas diminuta*, *Bradyrhizobium japonicum* (29), and *L. pneumophila* (32). Lipid A of *Campylobacter jejuni*, which is thus far the most thoroughly investigated representative of a GlcN3N-containing lipid A, is composed predominately of a hybrid GlcN3N-GlcN disaccharide, whereas GlcN3N-GlcN3N is found in minor LPS species (24). The GlcN3N-containing lipid A of *C. jejuni* has apparently similar endotoxic activities as those with the typical GlcN-containing backbone. Thus, there are so far no indications that the replacement of GlcN with GlcN3N in the lipid A backbone of *C. jejuni* influences its biological and physicochemical behavior (24, 33).

However, recently it has been reported that the LPS or lipid A from *L. pneumophila* can bind to and signal via TLR2 (34). The same TLR2-dependent signaling was observed for *Leptospira interrogans* LPS (35). As both lipid A's share an identical 4′-*P*-β-D-Glc*p*N3N-(1→6)-α-D-Glc*p*N3N-(1→*P* sugar backbone (36), we were curious to find out whether this structural feature might determine TLR2 *versus* TLR4 specific signaling. Since the LPS of *B. henselae* shares the same GlcN3N-GlcN3N-containing lipid A backbone, we examined whether the LPS of *B. henselae* can activate TLR2 signaling to a similar extend as *L. pneumophila* and *L. interrogans* LPS. We found that, in contrast to *L. pneumophila* LPS, the purified, protein-free LPS from *B. henselae* did not mediate any considerable TLR2 activation when tested in transiently transfected HEK cells. In agreement with what is stated above on the *C. jejuni* LPS, we thus conclude that the nature of the monosaccharide constituents in the lipid A backbone (GlcN *versus* GlcN3N) may not be critical for the endotoxic activity nor determines this structural feature TLR2-specific signaling.

The *B. henselae* lipid A is pentaacylated with each GlcN3N unit carrying one residue each of 3-hydroxydodecanoic and 3-hydroxyhexadecanoic acid. In addition, the non-reducing GlcN3N II residue carries one ester-linked long-chain fatty acid, which is either 25-hydroxypentacosanoic acid (26:0(25-OH)) or 27-hydroxyheptacosanoic acid (28:0(27-OH)). All *Rhizobiaceae* (*i.e.* the plant symbiontic rhizobia) and a few other *Rhizobiales*, including intracellular mammalian pathogens *Bartonella* and *Brucella*, examined to date contain a (ω-1)-hydroxylated long-chain fatty acids in their lipid A, likely reflecting their phylogenetic relationship (11, 29). However, be-

cause of a limited number of detailed structural studies on lipid A's from these organisms, and because of difficulties associated with analyzing the long-chain fatty acids, the location, stoichiometry, and type of attachment of these substituents is known only for a few species, including *Sinorhizobium* sp. NGR234 and *R. etli-R. leguminosarum* (30, 31). Interestingly, LPS of the unrelated intracellular pathogen *L. pneumophila* appears to have a related structure too (32) with similar long-chain fatty acids. However, *L. pneumophila* lipid A differs in the (ω-1)-substituent, which is either 28:0(27-keto) or 27:0(dioic) acid. In addition, *L. pneumophila* and *B. henselae* differ in the degree of lipid A acylation, *B. henselae* having a pentaacyl lipid A and *L. pneumophila* a hexaacyl lipid A (32).

These common structural features of LPS found among intracellular plant symbionts (*i.e.* the bacteroids of rhizobia) and intracellular mammalian pathogens *B. henselae* and *L. pneumophila* are expected to have implications for their biological activity. It is well known that enterobacterial lipid A with a reduced number of fatty acids, such as a pentaacyl lipid A lacking a secondary myristic acid (14:0) residue in *waaN*-mutant of *S. enterica* sv. Typhimurium, expresses significantly lower endotoxic activities in mouse peritoneal model (38). These *in vivo* data are in full agreement with previous results showing that the acylation pattern significantly influences the endotoxic activity in macrophages in various *in vitro* test systems (24, 33). In addition, it was postulated (32, 37) that long-chain fatty acids, as they are present in *L. pneumophila,* might be responsible for the failure to interact with CD14 and TLR4 (37). *B. henselae* lipid A possesses both features known to reduce endotoxicity, including a pentaacyl lipid A and a long-chain fatty acid. Consequently, it showed an at least 1,000-fold lower activity for signaling via TLR4 as compared with LPS from enteric bacteria, *e.g.* that from *S. enterica* sv. Friedenau. Similar to enterobacterial LPS, TLR4-mediated signaling by *B. henselae* LPS is CD14-dependent. It remains to be demonstrated whether the low endotoxic activity of *B. henselae* LPS results from a diminished interaction with CD14, with TLR4 or with both receptors.

In contrast to the TLR2 activity of *L. pneumophila* LPS (34), we could almost completely eliminate the TLR2 activity of *B. henselae* LPS upon further purification, especially with reduction of contaminating protein. The reason for the different TLR-specificity of the structurally related LPS from *L. pneumophila* and *B. henselae* remains elusive. As outlined above, the only difference in lipid A's of the two bacteria lies in the nature and number of fatty acids. In *L. pneumophila,* the main lipid A species carries six acyl groups, including *iso*- and dihydroxy-atty acids (*e.g. i*14:0(2,3-diOH)) and 28:0(27-keto) or 27:0(dioic) long-chain fatty acids (32). In contrast, *B. henselae* lipid A carries five acyl groups, no *iso*- and dihydroxy fatty acids, and 26:0(25-OH) or 28:0(27-OH) long-chain fatty acids. The importance of the number and the nature of acyl residues with respect to TLR2 and TLR4 signaling specificity is further supported with an example of lipid A's from *Porphyromonas gingivalis* (39) and *L. interrogans* (35, 36), which do not share any special structural relationship to each other in their acylation pattern. However, like *L. pneumophila,* they express TLR2--dependent rather than TLR4-dependent activity. Since *B. henselae* has a pentaacyl lipid A these data indicate that neither the structure of the lipid A backbone nor the degree of phosphorylation or acylation are sufficient to determine TLR2- and TLR4-specific activity. However, the fatty acid composition (*i.e.* the presence of hydroxy, olefinic, keto, and dihydroxy groups) represent distinct structural motifs of these lipid A and may thus contribute to determining TLR2 *versus* TLR4 specificity.

The carbohydrate portion of *B. henselae* rough-type LPS was found to consist of a branched trisaccharide, containing a glucose residue attached at position 5 of an α-(2→4)-linked Kdo disaccharide. A terminal Glc and the absence of L-*glycero*-d-*manno*-heptose and mannose (Man) (40) are remarkable features of this short-chain LPS core. While carbohydrate structures of a similar size are typically present in LPS of deep-rough mutants of bacteria otherwise containing O-polysaccharide chains, all isolates of the obligate intracellular pathogen *C. trachomatis* investigated so far contain an unbranched Kdo trisaccharide (41). Given the obligate intracellular life style of *C. trachomatis*, the short-chain carbohydrate moiety of LPS might help adaptation of the bacterium to intracellular life. The R-form LPS of the facultative intracellular pathogen *B. henselae* does not seem to result from a deep-rough mutation as at least one minor S-form LPS species is simultaneously produced in ATCC 49882^T and all other tested isolates (12). Heterogeneity of LPS in regard to the presence of an *O*-chain has previously been reported for the closely related species *B. bacilliformis* (14) and *B. quintana*,[2] as well as for some other rhizobacteria, including the plant symbionts *Bradyrhizobium japonicum* (28) and *Sinorhizobium* sp. NGR234 (31), and may be a typical feature of the *Rhizobiales*. Interestingly, synthesis of the LPS *O*-chain in *Sinorhizobium* sp. NGR234 is regulated differently in the free-living *versus* endosymbiotic state (31), suggesting that the relative proportion of R-form to S-form LPS species may be a critical factor for the facultative intracellular life style of this plant symbiont. In future it will be interesting to recognize whether the facultative intracellular pathogen *B. henselae* can regulate synthesis of different amounts of R-form *versus* S-form LPS species in response to environmental signals as a specific adaptation to the host.

Acknowledgments—We thank M. Röttgen for helpful discussions. We acknowledge H. P. Cordes, L. Brecker, and H. Lüthje for help with NMR and MALDI-TOF mass spectroscopy; K. Jakob and I. Goroncy for technical assistance; and H. Moll for expert GC-MS analysis. Dr. Yves Piemont is acknowledged for providing strain ISB 113. We thank Henri Saenz for critical reading of the manuscript.

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Phase-variable Expression of Lipopolysaccharide Contributes to the Virulence of *Legionella pneumophila*

By Edeltraud Lüneberg,* Ulrich Zähringer,‡ Yuriy A. Knirel,‡ Dorothee Steinmann,‡ Maike Hartmann,* Ivo Steinmetz,§ Manfred Rohde,‖ Jörg Köhl,§ and Matthias Frosch*

*From the *Institut für Hygiene und Mikrobiologie, Universität Würzburg, 97080 Würzburg, Germany; ‡Forschungszentrum Borstel, 23845 Borstel, Germany; §Institut für Medizinische Mikrobiologie, Medizinische Hochschule, 30625 Hannover, Germany; and ‖Gesellschaft für Biotechnologische Forschung, 38124 Braunschweig, Germany*

Summary

With the aid of monoclonal antibody (mAb) 2625, raised against the lipopolysaccharide (LPS) of *Legionella pneumophila* serogroup 1, subgroup OLDA, we isolated mutant 811 from the virulent wild-type strain RC1. This mutant was not reactive with mAb 2625 and exhibited an unstable phenotype, since we observed an in vitro and in vivo switch of mutant 811 to the mAb 2625–positive phenotype, thus restoring the wild-type LPS. Bactericidal assays revealed that mutant 811 was lysed by serum complement components, whereas the parental strain RC1 was almost serum resistant. Moreover, mutant 811 was not able to replicate intracellularly in macrophage-like cell line HL-60. In the guinea pig animal model, mutant 811 exhibited significantly reduced ability to replicate. Among recovered bacteria, mAb 2625–positive revertants were increased by fourfold. The relevance of LPS phase switch for pathogenesis of *Legionella* infection was further corroborated by the observation that 5% of the bacteria recovered from the lungs of guinea pigs infected with the wild-type strain RC1 were negative for mAb 2625 binding. These findings strongly indicate that under in vivo conditions switching between two LPS phenotypes occurs and may promote adaptation and replication of *L. pneumophila*. This is the first description of phase-variable expression of *Legionella* LPS.

Key words: *Legionella pneumophila* • LPS • phase-variation • serum resistance • virulence

Legionella pneumophila is the causative agent of Legionnaires' disease, a severe pneumonia with frequently fatal progression (1). The habitat of *Legionella* species are natural or man-made water reservoirs where the bacteria survive and multiply intracellularly in amebae (2–4) in tight association with biofilms (5–7). Infection of man occurs by inhalation of *Legionella*-containing aerosols, but person to person transmission has never been observed (1, 8). In the human lung attachment of *L. pneumophila* and internalization into alveolar-macrophages is mediated by the major outer membrane protein, MOMP,[1] the complement factors C3b and iC3b and the corresponding receptors (9, 10). In phagocytes fusion of *Legionella*-containing phagosomes with lysosomes is prevented and *L. pneumophila* survives and multiplies within macrophages (11, 12).

Several virulence factors of *L. pneumophila* have been identified and characterized. The macrophage infectivity potentiator protein (Mip) plays an important role in infection of macrophages, although its precise function is unclear (13–20). The products of the *icm* and *dot* loci are required for intracellular multiplication. Again, their role in the pathogenesis of disease is unresolved (21–25). Likewise, LPS of *L. pneumophila* is considered a factor mediating pathogenicity (8). It is the major immunodominant antigen and represents the basis for the classification of serogroups (26–29). In contrast to enterobacterial LPS activation it has been shown that *Legionella* LPS is able to activate both the classical and the alternative complement pathway (30). Due to the exceptional chemical structure of the *L. pneumophila* LPS, it is likely that this molecule participates in a number of essential legionellae capabilities, such as adaptation to

[1] *Abbreviations used in this paper:* BYCE, buffered CYE; CI, chemical ionization; CYE, charcoal yeast extract; EI, electron impact; GLC, gas–liquid chromatography; MAC, membrane attack complex; MOMP, major outer membrane protein; NHS, normal human serum; NMR, nuclear magnetic resonance; SG, serogroup.

This work is dedicated to Dieter Bitter-Suermann on the occasion of his 60th birthday.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/98/07/49/12 $2.00
Volume 188, Number 1, July 6, 1998 49–60
http://www.jem.org

various environmental challenges (31). The *L. pneumophila* serogroup (SG) 1 (strain Philadelphia) LPS differs from that of other Gram-negative bacteria in that its lipid A section consists of long chain fatty acids which may account for the weak endotoxicity of the molecule (31). The O-specific chain is composed of an α-(2→4) interlinked 5-acetamidino-7-acetamido-8-*O*-acetyl-3,5,7,9-tetradeoxy-L-*glycero*-D-*galacto*-nonulosonic acid (legionaminic acid) homopolymer. This unusual sugar molecule completely lacks free hydroxyl groups and is thus very hydrophobic (31, 32). In addition, an isomer of legionaminic acid hypothesized to be a terminal sugar of the LPS O-chain has been recently detected (33). The outer core oligosaccharide also exhibits hydrophobic properties (34). Based on these findings, it can be assumed that *L. pneumophila* possesses a hydrophobic cell surface that may support concentration of the bacterium in aerosols as well as adherence to host cells (31, 35).

To further elucidate the role of the LPS molecule and the surface properties of *L. pneumophila* in adaptation to various exogenous conditions, we raised mAb against the LPS of *L. pneumophila* SG 1 (subgroup OLDA). In this study, we describe mAb 2625 which binds to this LPS. Moreover, we show that the O-chain as well as the core are required for binding of mAb 2625. With the aid of mAb 2625, we isolated an LPS mutant from the virulent patient isolate RC1 (subgroup OLDA). Here we report for the first time that the LPS structure appears to be a virulence determinant of *L. pneumophila* and that expression of *L. pneumophila* LPS occurs in a phase-variable manner.

Materials and Methods

Bacterial Strains and Cultivation. *L. pneumophila* SG 1 strain RC1 (OLDA), a clinical isolate, was a generous gift from B. Wright (Rigshospitalet, Copenhagen, Denmark). All other *Legionella* strains were obtained from the American Type Culture Collection (Rockville, MD) and the National Collection of Type Cultures (London, UK), respectively. Strains and sources are listed in Table 1. *Legionella* strains were cultivated on charcoal yeast extract (CYE) agar supplemented with buffered charcoal yeast extract (BCYE) growth supplement and MWY selective supplement (Unipath-Oxoid, Wesel, Germany). Plates were incubated at 37°C under 5% CO_2 for 48–72 h unless otherwise stated. Propagation in liquid media (1% wt/vol yeast extract supplemented with BCYE growth supplement) was carried out at 37°C under constant agitation.

Pseudomonas aeruginosa (ATCC 49266) was obtained from the American Type Culture Collection. The following strains were isolates from the Institut für Medizinische Mikrobiologie (Medizinische Hochschule Hannover, Germany): *P. fluorescens*, *Bordetella pertussis*, *Acinetobacter lwoffii*, and *Escherichia coli*.

Production of Monoclonal Antibodies. 6-wk-old female BALB/c mice (Zentralinstitut für Versuchstierkunde, Hannover, Germany) were immunized intraperitoneally for four times once a week with 2×10^8 *L. pneumophila* SG 1 strain RC1 viable cells as previously described (36). Before injection, bacteria were passaged once in a guinea pig as described below. At the end of the immunization regimen, mice were splenectomized and the spleen cells were fused with X63-Ag8.653 myeloma cells as described elsewhere (37). The culture supernatant fluids of growing clones were screened by ELISA with whole *L. pneumophila* SG 1 (strain RC1) cells as antigens. The resulting hybridomas were cloned by limiting dilution.

Immunoelectron Microscopy. Bacteria were fixed with 0.5% formaldehyde and 0.2% glutaraldehyde (final concentrations) in 0.1 M PBS for 1 h on ice. After three washes with 0.1 M PBS containing 10 mM glycine to block free aldehyde groups, the cells were embedded by progressively lowering the temperature with Lowicryl K4M resin (38). The following modifications of the method were made: (*a*) after dehydration in 10% ethanol, the samples were treated with 0.5% uranyl acetate in 10% ethanol for 1 h on ice; (*b*) the infiltration step with 1 part ethanol and 1 part K4M resin was performed overnight; (*c*) the infiltration step with one part ethanol and two parts K4M resin lasted for 12 h; and (*d*) infiltration with pure K4M resin lasted for 2 d. After polymerization of the samples for 2 d at −35°C, samples were trimmed and polymerized for another day at room temperature. Ultrathin sections were incubated overnight with 200 μg of IgG per ml of mAb 2625 or mAb LPS-1 (39) at 4°C. mAb LPS-1 was purchased from Progen (Heidelberg, Germany). After washing with 0.1 M PBS, sections were incubated with protein A–gold complexes (10-nm diam; concentration giving an A_{520} of 0.02). The sections were subsequently rinsed with 0.1 M PBS containing 0.01% Tween 20 and then with distilled water. After air drying, the sections were counterstained with 4% aqueous uranyl acetate (pH 4.5) for 5 min. Samples were examined with a Zeiss EM 910 electron microscope at an acceleration voltage of 80 kV at calibrated magnifications.

Western Blot. 1 ml of bacterial cell suspensions (OD_{550nm} 1.2) were centrifuged and the resulting pellet was resuspended in 100-μl sample solution (20% glycerol, 3% sodium dodecyl sulfate, 3% 2-mercaptoethanol, 1% bromphenol blue). The suspensions were heated to 100°C for 5 min before 5-μl aliquots were applied to 12.5% polyacrylamide gels. To ensure equal protein concentrations in each lane, control gels were stained with Coomassie Blue dye (Sigma Chemical Co., Deisenhofen, Germany). For analysis of LPS samples, 2 μg purified LPS was applied to gels after boiling in sample solution. Western blotting onto nitrocellulose filters was carried out as described by Towbin et al. (40). Filter membranes were blocked with 3% dried milk powder suspended in PBS. Immunostaining was performed with mAb 2625 and mAb LPS-1 (39), respectively, and subsequent incubation with alkaline phosphatase-labeled goat anti-mouse immunoglobulin (Dianova, Hamburg, Germany).

Colony Blot. Nitrocellulose filters were soaked in sterile PBS before colonies grown on BCYE agar were blotted. Filter membranes were then placed for 5 minutes on paper filter sheets (Schleicher and Schuell, Dassel, Germany) soaked with 70% ethanol. Nitrocellulose filters were air-dried at room temperature and subsequently blocked with 3% dried milk powder suspended in PBS. Immunostaining of the filters was performed as described above.

Extraction of LPS and Isolation of O-Chain Polymers. LPS of *L. pneumophila* SG 1 strain RC1 (subtype OLDA) and mutant 811 was isolated from dry cells by a modified phenol-chloroform-petroleum ether procedure as described (32, 41). Starting from washed and enzymatically degraded dried cells excellent yields were obtained ranging between 8.1% (wt/wt) for the wild-type and 9.9% (wt/wt) for the mutant, respectively. LPS (155 mg of the wild-type, 144 mg of the mutant) was degraded with 0.1 M NaOAc-HOAc buffer (pH 4.4, 30 ml) at 100°C for 4 h and the resulting precipitate was removed by centrifugation. The supernatant was freeze-dried and fractionated by gel permeation chro-

Table 1. *Legionella Strains Used in this Study and Indication of the Source*

Strain	Source	Strain	Source
L. pneumophila SG 1 (OLDA)	ATCC 43109	*L. pneumophila* SG 1 (Oxford)	ATCC 43110
L. pneumophila SG 1	ATCC 33152	*L. pneumophila* SG 1	ATCC 33153
L. pneumophila SG 1	ATCC 43108	*L. pneumophila* SG 1	ATCC 43112
L. pneumophila SG 1	ATCC 43106	*L. pneumophila* SG 1	ATCC 43107
L. pneumophila SG 1	NCTC 11191	*L. pneumophila* SG 1	NCTC 11193
L. pneumophila SG 1	NCTC 11201	*L. pneumophila* SG 1	NCTC 11231
L. pneumophila SG 1	NCTC 11378	*L. pneumophila* SG 1	NCTC 11404
L. pneumophila SG 2	ATCC 33154	*L. pneumophila* SG 3	ATCC 33155
L. pneumophila SG 4	ATCC 33156	*L. pneumophila* SG 5	ATCC 33216
L. pneumophila SG 6	ATCC 33215	*L. pneumophila* SG 7	ATCC 33823
L. pneumophila SG 8	ATCC 35096	*L. pneumophila* SG 9	ATCC 35289
L. pneumophila SG 10	ATCC 43283	*L. pneumophila* SG 11	ATCC 43130
L. pneumophila SG 12	ATCC 43290	*L. pneumophila* SG 13	ATCC 43736
L. pneumophila SG 14	ATCC 43703	*L. anisa*	ATCC 35291
L. cherrii	ATCC 35252	*L. erythra*	ATCC 35303
L. birminghamensis	ATCC 43702	*L. bozemanii*	ATCC 33217
L. dumoffii	ATCC 33279	*L. gormanii*	ATCC 33297
L. micdadei	ATCC 33204		

matography on a Sephadex G-50 (S) column (2.5 × 50 cm; Pharmacia Biotechnology Inc., Freiburg, Germany) using a pyridinium acetate buffer (pH 4.5) and monitoring with a Knauer differential refractometer. Appropriate fractions were pooled and lyophilized.

SDS-PAGE and LPS Silver Staining. SDS-PAGE was carried out in 14% polyacrylamide gels using Mini-Protean II system (Bio-Rad Laboratories, München, Germany). LPS bands were visualized by the silver-staining technique as described elsewhere (42).

Chemical LPS Analysis by Gas–Liquid Chromatography and Nuclear Magnetic Resonance. Gas–liquid chromatography (GLC) was performed with a Varian Model 3700 chromatograph equipped with a capillary column of SPB-5 using a temperature gradient 150→320°C at 5°C/min. GLC-mass spectrometry in both chemical ionization (CI, with ammonia) and electron impact (EI) modes was carried out with a Hewlett-Packard Model 5989 instrument equipped with a capillary column of HP-1 under the same chromatographic conditions as in GLC. Monosaccharides were analyzed by GLC after methanolysis with 2 M HCl/MeOH (120°C, 16 h) and acetylation with Ac_2O in pyridine (70°C, 0.5 h). ^1H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were obtained with a Bruker AM-360 spectrometer for solutions in D_2O at 60°C with acetone (δ_H 2.225, δ_C 31.45) as internal standard. Standard Bruker software was used in all ^1H- and ^{13}C-NMR experiments.

Competition ELISA. 1 μg purified LPS from *L. pneumophila* strain RC1 in 20 μl 0.2 M sodium carbonate buffer, pH 9.6, per well was adsorbed to microtiter plates (Microlon, Greiner, Nürtingen, Germany) overnight at 4°C. Plates were subsequently blocked with 2% (wt/vol) dried milk powder in PBS for 1 h at room temperature. mAbs 2625 and LPS-1 (39), respectively, were preincubated with carbohydrate fractions from wild-type strain RC1 and mutant strain 811, respectively. These sugar fractions containing O-chain polysaccharide with attached core oligosaccharide were obtained from gel permeation chromatography as described above. The polysaccharides were redissolved in distilled water to a concentration of 50 μg/μl. 1 μl of serial dilutions of carbohydrate fractions was added to 19 μl dilutions of mAb 2625 and mAb LPS-1, respectively. Before addition of the sugars the antibodies were diluted to a suitable concentration in PBS. In control reactions 1 μl distilled water was added to the antibody dilutions instead of carbohydrate solutions. Coincubation of mAbs and carbohydrate fractions was performed at 4°C overnight. After blocking and three washing steps with PBS, microtiter plates were incubated with the antibody–carbohydrate mixture for 2 h at room temperature and subsequently washed with PBS for three times. Detection was carried out by incubation of the plates with peroxidase-labeled goat anti-mouse antibody (Dianova) for 1 h at room temperature. After three washing steps as before the substrate H_2O_2 and azino-di-ethylbenzthiazolinsulfonate (ABTS) was added and after a 30-min incubation at room temperature absorbance was determined in a microplate reader at 405 nm. Inhibition of antibody binding was calculated as percentage of the control reactions (no carbohydrates added). All reactions were carried out in duplicates.

Bactericidal Assay. For investigation of serum resistance of *L. pneumophila* wild-type strain RC1 and mutant strain 811 normal human serum (NHS) was obtained from 10 healthy volunteers. Blood was allowed to clot for 30 min at room temperature. After centrifugation for 5 min at 2,000 *g*, the sera were pooled, quick-frozen in liquid nitrogen and stored at −80°C. Pool serum was negative for anti-*L. pneumophila* antibodies as determined by standard diagnostic serology methods (immune fluorescence test). Bacteria were plated on BCYE agar from frozen stocks and suspended in 0.9% saline after 40 h of incubation. Optical density was determined at 600 nm and bacterial suspensions were appropriately diluted in 0.9% saline. 40% NHS was incubated with 10^6 bacteria in a final reaction volume of 1 ml. The reaction mixture was incubated at 37°C in a water bath and stopped on ice at 0, 15,

30, and 60 min, respectively. Appropriate dilutions were plated on BCYE agar.

Infection of HL-60 Cells with L. pneumophila. Infection of HL-60 cells was performed essentially as described (25, 43). Human macrophage-like cell line HL-60 was propagated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (GIBCO BRL, Eggenstein, Germany). Differentiation of the cells was induced with PMA (Sigma Chemical Co.) at a final concentration of 10^{-8} M. After 48 h of incubation with PMA cells were washed and 0.5×10^6 adherent HL-60 cells per well were infected with 10^4 bacteria of the appropriate *L. pneumophila* strain. Bacteria were plated on BCYE agar from frozen stocks and harvested after 40 h incubation. The optical density of bacterial suspensions in 0.9% saline was determined at 600 nm and appropriate dilutions were prepared in RPMI medium. For determination of intracellular multiplication, at 0, 24, 48, and 72 h after infection culture supernatants were removed, HL-60 cells were lysed by suspending in ice-cold water. Suspended cells and supernatant were pooled and aliquots of serial dilutions were plated on BCYE agar.

In a second assay, extracellular remaining bacteria were killed by adding gentamicin (GIBCO BRL) to a final concentration of 40 μg/ml after 2 h of coincubation of HL-60 cells and bacteria. After a 2-h incubation time with gentamicin, cells were washed again and incubated in antibiotic-free medium. Under these conditions, HL-60 were infected with 10^6 bacteria per well. At time intervals of 0, 24, 48, and 72 h after gentamicin removal, appropriate dilutions were plated for determination of intracellular multiplication.

Intratracheal Infection of Guinea Pigs. Male and female guinea pigs strain 2BS (400–600 g) obtained from the Zentrales Tierlaboratorium (Medizinische Hochschule Hannover, Germany) were infected with *L. pneumophila* by intratracheal application of the bacteria as described (15, 44). Animals were anesthetized by intramuscular injection of a mixture of 10 μl ketamine (10% solution) and 10 μl xylazinhydrochloride (2% solution) per 100 g body weight. Subsequently, a small skin incision was made in the ventral neck and the bacterial suspension was injected into the exposed trachea. Before inoculation, the appropriate *L. pneumophila* strains were plated on BCYE agar from frozen stocks and were incubated for 40 h. Bacteria were harvested and suspended in 0.9% saline to a concentration of 10^8/ml. 0.3 ml (corresponding to 3×10^7 CFU) of this suspension was injected into the tracheal lumen with a 24-gauge needle and the incision was closed with sutures. In previous experiments, 3×10^7 CFU had been determined as the 50% lethal dose (LD_{50}) of strain RC1 to guinea pig strain 2BS within 3–5 d after infection. After the infection, animals were observed several times daily for signs of illness and respiratory disease. 48 h after infection animals were killed and the lungs were removed. Lung tissue was homogenized in 0.9% saline and aliquots of serial dilutions of the lung suspensions were plated on BCYE agar. For determination of recovered *Legionella*, CFU were counted after 72 h incubation. Aliquots of the lung suspensions were also plated on blood agar to control for contaminating bacteria. Binding of injected and recovered bacteria to mAb 2625 was in all experiments monitored by colony blot assay. Animal experiments were carried out with the permission and according to the guidelines of local authorities.

Results

Characterization of mAb 2625. To generate mAbs, mice were immunized with whole cells of *L. pneumophila* SG 1 strain RC1 (OLDA). Before the immunization, strain RC1 was subjected to a single guinea pig passage. Screening of hybridoma supernatants in an ELISA with the same strain used for immunization led to the isolation of IgG3 mAb 2625. The reactivity of mAb 2625 was not eliminated after treatment with proteinase K, suggesting that a carbohydrate epitope was recognized. Binding of mAb 2625 occurred exclusively in strains belonging to the OLDA and Oxford subgroups, of *L. pneumophila* SG 1, respectively, as determined by ELISA and Western blot analysis. There was no binding reaction observed in any other of the 14 tested SG 1 strains. This was also the case for type strains from serogroup 2 to 14. Likewise, eight non-*pneumophila* strains as well as several bacteria from other genera were tested and did not bind to mAb 2625. All of the investigated strains are listed in Materials and Methods.

In electron microscopy experiments, the epitope on the antigen bound by mAb 2625 could be localized to the cell surface of *L. pneumophila* (Fig. 1). In Western blot analysis of whole cell lysates and purified LPS, mAb 2625 exhibited a ladder-like binding pattern characteristic of LPS (Fig. 2 *A*, lanes *1* and *3*). With the aid of molecular mass standards for SDS-PAGE, the ladder-like bands were estimated to be in the range of 30–65 kD. This molecular mass range does not correspond to the molecular mass of *L. pneumophila* LPS, but permitted a relative comparison of LPS banding patterns in Western blots. In control experiments, we incubated Western blots with mAb LPS-1 (39), an mAb specific to serogroup 1 of *L. pneumophila*. As shown in Fig. 2 *B* (lanes *1* and *3*), LPS-1 exhibited a ladder-like banding pattern in the range of 20–30 kD and in addition, faint bands in the range of 35–45 kD. This binding pattern corresponds well to the image of *L. pneumophila* LPS bands which become visible after silver staining (Fig. 3). From the binding characteristics of mAb 2625, that is the banding pattern in a higher molecular mass range than observed with mAb LPS-1, we conclude that mAb 2625 binds a conformational epitope, which is not generated before a distinct O-chain length is achieved.

Isolation of LPS-Mutant Strain 811. Next, we wondered whether mAb 2625–reactive epitope is present on all bacteria within a population. Therefore, we performed colony blots on the virulent *L. pneumophila* strain RC1, a clinical isolate of the OLDA subtype of serogroup 1, with mAb 2625. Using this strategy, we could show that mAb 2625–negative colonies could be detected in a frequency of 10^{-4}. We isolated one of the mAb 2625–negative colonies for further analysis. Interestingly, the phenotype of this LPS variant, termed 811, was unstable and exhibited a remarkable switching back to the LPS phenotype of the parent strain. This could be shown using mAb 2625. When a single colony of the mutant 811 with mAb 2625 nonreactivity confirmed by colony blot analysis was restreaked on BCYE agar, 80–100% of the grown colonies were again positive for binding of mAb 2625. Colony morphology of mutant strain 811 did not exhibit any differences compared with that of the wild-type strain, but the colony material appeared extremely viscous and sticky. By repeating the pro-

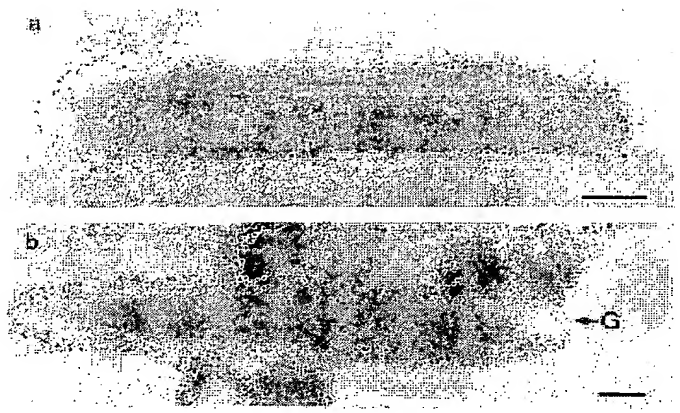

Figure 1. Immunoelectron microscopy of *L. pneumophila* SG 1 strain RC1 (OLDA). Staining of sections was performed with mAb 2625 (*a*). In control experiments sections were immunostained with mAb LPS-1 (39), which binds specifically to LPS of *L. pneumophila* SG 1 (*b*). *G*, gold-particle. Bar, 0.25 μm.

cedure of colony blots and restreaking single mAb 2625–negative colonies (three passages), we could reduce the proportion of mAb 2625–positive (wild-type) colonies of the mutant 811 to ~10%. For all experiments described in the following paragraphs, the mutant 811 was subjected to this treatment. The ~10% portion of wild-type cells in the mutant 811 population was controlled in all experiments and confirmed by colony blot analysis with mAb 2625. Mutant 811 replicated with the same growth rate in liquid media as the wild-type RC1. As could be expected from colony blot analysis, mutant 811 did not bind mAb 2625 in Western blot analysis (Fig. 2 *A*, lanes *2* and *4*). The faint bands visible in lane *2* of Fig. 2 *A* are due to ~10% wild-type cells present in the bacterial suspension. In contrast, mAb LPS-1 revealed enhanced binding to mutant 811 in comparison to the parental wild-type strain RC1, resulting in a more sensitive staining of high molecular mass LPS populations. It is conceivable that LPS of mutant 811 is altered in a way that the epitope bound by mAb LPS-1 becomes more accessible and antibody binding is thereby promoted.

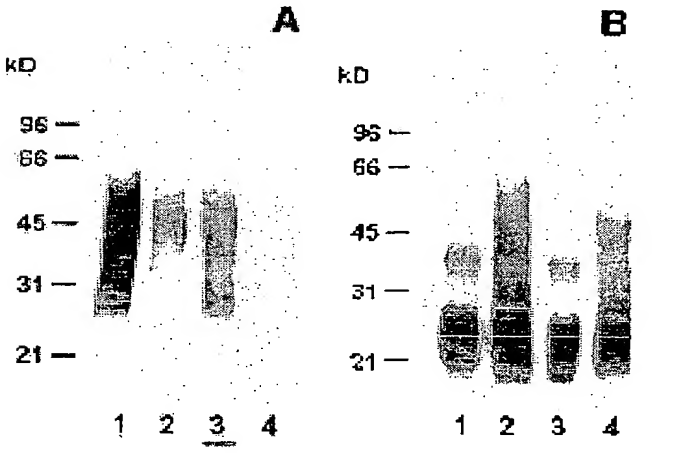

Figure 2. Western blot analysis of wild-type strain RC1 and mutant 811 with mAb 2625 (*A*) and mAb LPS-1 (*B*), respectively. Lane *1*, wild-type RC1 whole cell lysate; lane *2*, mutant 811 whole cell lysate; lane *3*, wild-type RC1 2 μg purified LPS; lane *4*, mutant 811 2 μg purified LPS. Numbers on the left side indicate molecular masses of a standard protein marker. The molecular mass of *L. pneumophila* LPS does not correspond to that of the marker proteins, but determination of a relative range of LPS bands was achieved by this method.

Chemical Analysis of the LPS O-Chain of OLDA Wild-type RC1 and Mutant 811. To determine the chemical alteration in LPS composition of mutant 811, which was indicated by the antibody binding characteristics, we analyzed the LPS O-chain as well as the core oligosaccharide of both strains. For this purpose, the LPS from strains RC1 and mutant 811 was isolated. In Western blot analysis, purified LPS from both strains exhibited the same binding characteristics with mAb 2625 and mAb LPS-1 as has been determined for cell lysates of the strains (Fig. 2, *A* and *B*, lanes *3* and *4*). As recently published, the core oligosaccharide of *L. pneumophila* SG 1 strain Philadelphia is composed of Rha/QuiNAc/GlcNAC/Man/Kdo in a relative ratio of ~2:1:2:2:2 (34, 45). Analysis of the core sugar components of strains RC1 and mutant 811 by GLC-MS revealed that the same sugars were present in both strains. However, complete analysis of the core structure has not yet been accomplished. In particular, modification of sugars, conformational structures, and ketosidic linkages within the core remain to be established. Chemical analysis of the carbohydrate composition of the O-chain was performed by ^{1}H- and ^{13}C-NMR spectra analysis. It was found that the O-specific chain was composed of a homopolymer of α-(2→4) interlinked 5-acetamidino-7-acetamido-3,5,7,9-tetradeoxy-L-*glycero*-D-*galacto*-nonulosonic acid (8-de-*O*-acyl derivative of legionaminic acid). No structural difference was observed between the 8-*O*-deacetylated legionaminic acid in the two strains investigated. In addition, length distribution of the LPS O-chain was identical in wild-type RC1 and mutant 811, as was determined by SDS-PAGE and gel permeation chromatography. SDS-PAGE of purified LPS from RC1 and 811 revealed a ladder-like banding pattern with a bimodular distribution of O-chain length which is typical for *Legionella* LPS (Fig. 3). No difference in banding pattern was observed between wild-type and mutant. Moreover, both strains exhibited a banding pattern very similar to that of *L. pneumophila* SG 1 strain Philadelphia. In conclusion, no difference in O-chain structure and length was observed between wild-type RC1 and mutant 811, further confirming chemical data indicating the O-chain not to be changed in the mutant.

Epitope Mapping of mAb 2625 and mAb LPS-1. To determine the region of the LPS molecule where a structural difference between wild-type RC1 and mutant 811 could be located, we attempted to map the epitopes bound by mAb 2625 and mAb LPS-1, respectively. For this purpose we investigated the binding capacity of sugar fractions obtained from the gel filtration assay. Before separation on Sephadex columns, the lipid A moiety had been removed from LPS by mild acid hydrolysis. After gel filtration three

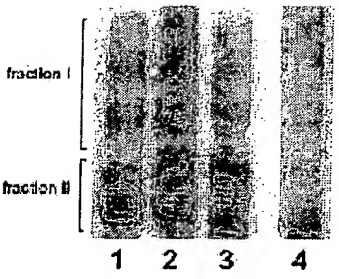

Figure 3. SDS-PAGE and silver staining of LPS from *L. pneumophila* SG 1. 2 μg purified LPS were applied to each lane. Lane *1*, mutant 811; lane *2*, wild-type RC1; lane *3*, strain Philadelphia. All strains exhibit a characteristic bimodular distribution of LPS O-chain representing 10–35 and 45–100 carbohydrate units, respectively. On the left side, LPS bands corresponding to fractions I and II of the gel filtration on Sephadex columns are indicated. These fractions were employed for epitope mapping by competition ELISA. As a control, 2 μg purified LPS from *P. aeruginosa* subgroup Fisher 2 was applied to lane *4*.

fractions of O-chain with attached core oligosaccharide were eluted from the column: fraction I represented the long O-chain (45-100-mer); fraction II the short O-chain (10-35-mer); and fraction III the isolated core oligosaccharide. The LPS bands separated by SDS-PAGE, which correspond to fractions I and II, are indicated in Fig. 3. All fractions eluted from the column contained the carbohydrate moiety of LPS (O-specific chain attached to the core) alone and therefore could not be analyzed by Western blot or conventional ELISA techniques. For this reason we performed a competition ELISA which is based on the principle of inhibition of antibody binding to LPS by LPS carbohydrate fractions. Purified LPS from *L. pneumophila* SG 1 wild-type RC1 was adsorbed to microtiter plates. mAb 2625 and LPS-1, respectively, were preincubated with serial dilutions of sugar fractions obtained from Sephadex gel filtration. In this way we investigated fractions I and II from wild-type RC1 and mutant 811 as well as the core portion of both strains. The results of the competition ELISA are illustrated in Fig. 4. Binding of mAb 2625 is inhibited in a concentration dependent manner by carbohydrates from fractions I and II of wild-type RC1, but not by the core oligosaccharide, indicating that the epitope bound by mAb 2625 involves the O-specific chain. It is noteworthy, that carbohydrates from fraction II exhibited a decreased binding to mAb 2625 in comparison to those from fraction I. O-chain molecules of the required length are presumably only present as a minor part of fraction I. For mutant 811, weak binding of carbohydrates from fraction I to mAb 2625 is due to wild-type bacteria (~10%, see above) that are inherent to the unstable character of 811. Inhibition of binding of mAb LPS-1 occurred in a likewise dose-dependent way. Carbohydrates from fractions I and II, as well as the core fractions from wild-type RC1 and mutant 811, were bound by mAb LPS-1. As already shown in Western blot analysis (see Fig. 2), binding of mAb LPS-1 to 811 is increased in comparison to wild-type RC1 (Fig. 4, *C* and *D*). In conclusion, mAb 2625 binds the LPS O-chain, but not the isolated core oligosaccharide. However, we cannot exclude that the core moiety is also required for mAb 2625 binding, since carbohydrates from fractions I and II contain the core portion as well as the O-chain. Since no differences in the O-antigen composition by chemical analysis could be observed, we conclude that the epitope bound by mAb 2625 is a conformational epitope which involves both the O-chain and the core. In contrast, the epitope bound by mAb LPS-1 is clearly located in the core oligosaccharide, and the O-chain is not required for binding. Together with the results obtained from chemical analysis, these data strongly support the idea, that alterations in the core oligosaccharide of mutant 811 are responsible for loss of mAb 2625 binding and increased binding to mAb LPS-1.

Intracellular Replication of RC1 and 811 in HL-60 Cells. Virulence of mutant 811 in comparison to the parent strain RC1 was determined by infection of human macrophage-like cell line HL-60 and determination of CFU on days 1 to 3 after infection. 2×10^6 HL-60 cells were infected with 10^4 bacteria from frozen stocks. Wild-type strain RC1 proved to be virulent and replicated in HL-60 cells by 2 orders of magnitude within 72 h (Fig. 5). A mAb 2625–positive revertant of mutant 811 (811-rev.) served as a control in these experiments. It is well known that virulence of pathogenic bacteria is attenuated or even abolished after laboratory passage on artificial media, strain 811-rev. was therefore employed to identify those effects due to agar passage. In comparison with the animal passaged wild-type strain RC1, 811-rev. exhibited a slightly reduced growth rate, but proved to be virulent (Fig. 5). In contrast, mutant 811 failed to replicate intracellularly in HL-60 cells. The number of recovered bacteria at all time intervals remained in the range of the inoculum (Fig. 5). In conclusion, wild-type RC1 proved to be able to replicate in HL-60 cells, whereas mutant 811 showed no replication and consequently proved to be avirulent. Moreover, by investigating 811-rev., it became evident that switching of 811 to the wild-type LPS-phenotype (mAb 2625 binding) also restored virulence. These findings indicate that alteration of LPS carbohydrate moiety was the only mutation that had occurred in 811. When gentamicin was added to kill extracellular bacteria after 2 h of coincubation of host cells and bacteria, mutant 811 was found intracellularly to approximately the same extent as wild-type RC1 and 811-rev. (Fig. 6). These data show that mutant 811 entered HL-60 cells as efficiently as the wild-type strain, but was unable to replicate in the host cell. Colony blot analysis of mutant 811 revealed that the percentage of mAb 2625-positive revertants among bacteria recovered from the HL-60 infection assays was identical to that of the inoculum.

Investigation of Serum Resistance. To determine the bactericidal activity of complement present in NHS on wild-type RC1, mutant 811 and 811-rev., we performed bactericidal assays as described in Materials and Methods. After a 1-h incubation in 40% serum, number of bacteria from wild-type strain RC1 declined by <1 log. Thus, wild-type RC1 was not completely, but almost serum resistant. In contrast, the number of viable bacteria from mutant 811 declined by 3 logs (from 6.1 $\log_{10}$ to 3.2 $\log_{10}$) within 15 min incubation in 40% NHS (Fig. 7). After 30 min no viable bacteria were recovered (detection limit 10^2 CFU/ml). Therefore, mutant 811 was serum-sensitive and the sensi-

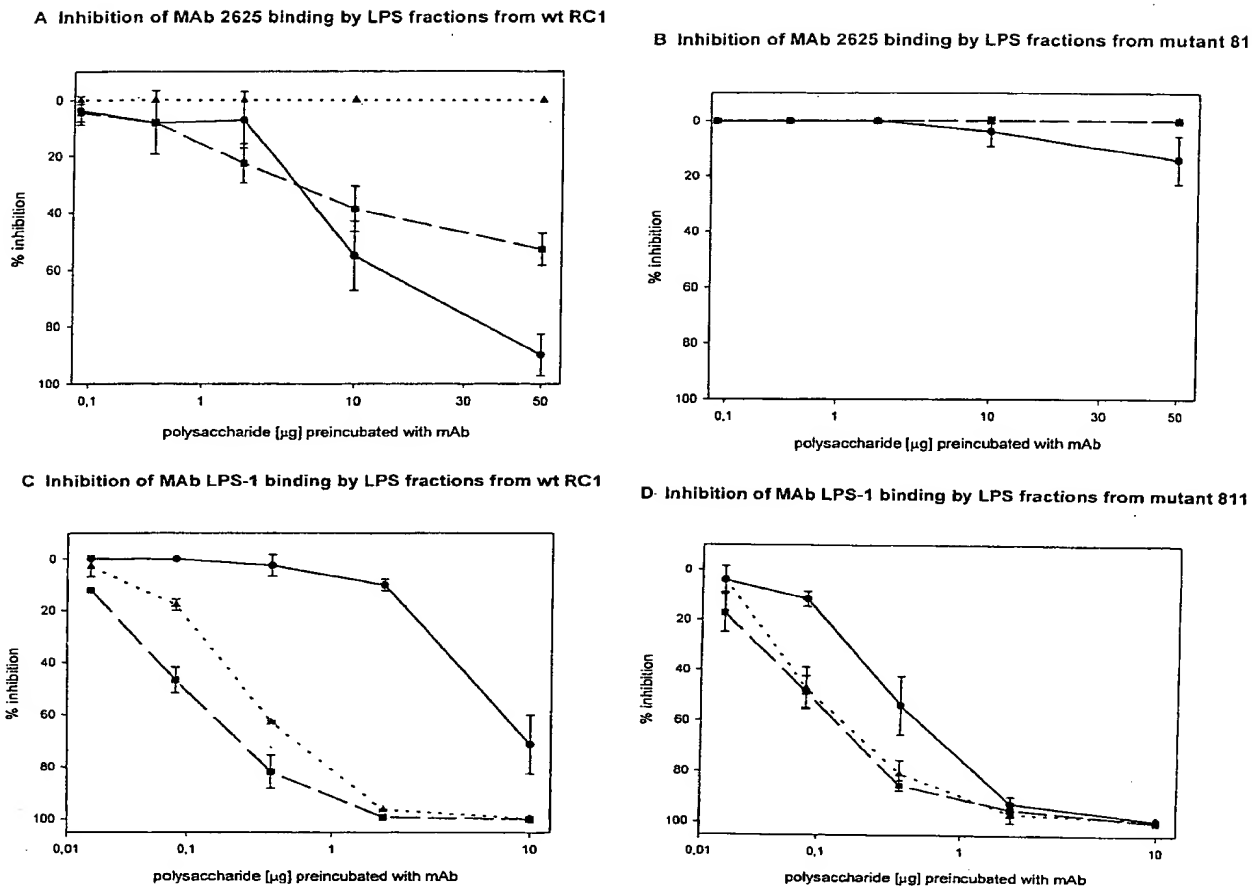

Figure 4. Epitope mapping of mAb 2625 (*A* and *B*) and mAb LPS-1 (*C* and *D*), respectively. Inhibition of binding of mAb 2625 and mAb LPS-1 to LPS from wild-type RC1 by LPS fractions from wild-type RC1 (*A* and *C*) and mutant 811 (*B* and *D*) was analyzed by competition ELISA. Percentage of inhibition is given as means of duplicate values. Fraction I is represented by circles, fraction II by squares and the core portion by triangles.

tivity is related to an altered LPS conformation. Incubation of 811-rev. in 40% NHS revealed that the number of viable bacteria was reduced by 2 logs within 1 h (Fig. 7). These findings indicate that agar passage does indeed have an influence on serum resistance, even though its effect is minor. From the results of the bactericidal assays, we conclude that the alteration in LPS carbohydrate moiety of mutant 811, which is presumably located in the core oligosaccharide, results in deprivation of resistance against serum complement activity. In addition, our data show that serum resistance of *L. pneumophila* is mediated by the LPS carbohydrate moiety.

In Vivo Virulence of Wild-type RC1 and Mutant 811. Next, we were interested to study virulence of mutant 811 under in vivo conditions in the guinea pig animal model. Animals ($n = 4$ for each bacterial strain) were infected by intratracheal injection of bacteria. On day 2 after infection, animals infected with the wild-type strain RC1 exhibited signs of severe illness as fever, ruffled fur, almost no motion and reaction and respiratory distress. Animals were killed on day 2 after infection and lungs were removed. The lungs appeared greatly enlarged and were completely hemorrhagic. After homogenization of the lungs, aliquots of the homogenates were plated for determination of the number of bacteria. 6.5×10^9 (mean value) bacteria were recovered from the animals (Fig. 8). The control blood agar plates were found to be sterile. In contrast, the animals infected with the mutant strain 811 showed moderate signs of illness, such as elevated body temperature and limited motion, but did not show signs of respiratory distress. These animals were also killed on day 2 after infection. Lungs were only slightly enlarged and hemorrhagic patches were visible, but were not distributed over the entire lung tissue. 1.8×10^8 viable bacteria (mean value) were recovered from the animals, a significantly lower number than was determined for the wild-type strain (Fig. 8). Interestingly, colony blot analysis of strain 811 isolated from animal lungs revealed that 35% of the recovered bacteria were positive for mAb 2625 binding, whereas only 8% of the inoculated bacteria were mAb 2625-positive. These results indicate that under in vivo selective pressure the 2625–positive phenotype (wild-type phenotype) is evidently advantageous over the

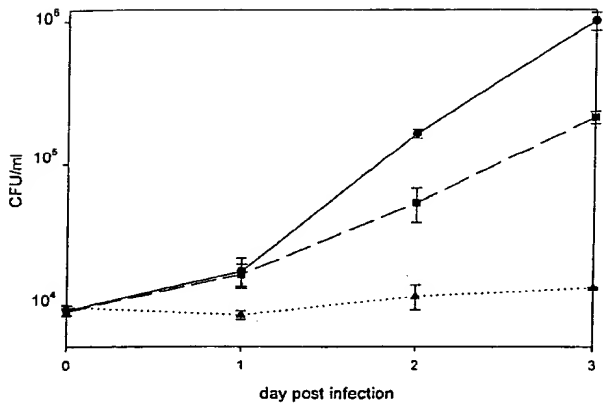

Figure 5. Infection of HL-60 cells with *L. pneumophila* strains RC1 (*circles*), 811 (*triangles*), and 811-rev. (*squares*). CFU were determined at 24, 48, and 72 h post infection and are shown as means of duplicates.

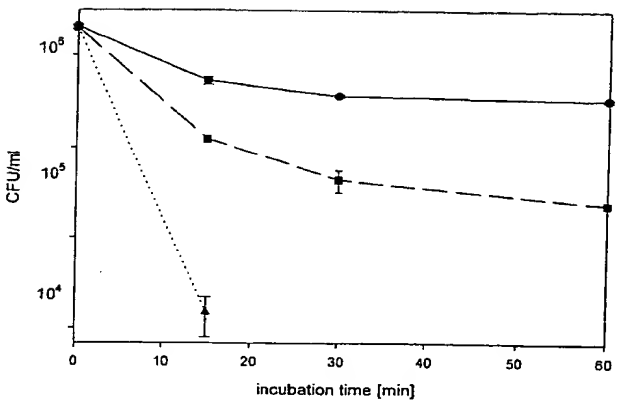

Figure 7. Bactericidal assay for determination of the lytic effects of serum complement on wild-type RC1 (*circles*), mutant 811 (*triangles*) and 811-rev. (*squares*). Bacteria were incubated with 40% normal human serum at 37°C. Aliquots of the reactions were plated at different time intervals for determination of viable bacteria. CFU are shown as mean of duplicates.

mAb 2625–negative phenotype of mutant 811. It remains unclear if a preferential replication of the mAb 2625–positive portion of mutant 811 occurred or if switching back to the wild-type phenotype is promoted under in vivo conditions.

On the other hand, for the wild-type strain RC1 we observed a significantly increased switching frequency under in vivo conditions to the mAb 2625–negative phenotype. A frequency of 5×10^{-2} among recovered bacteria from guinea pig lungs was determined by colony blot assay, whereas mAb 2625–negative clones could be detected among agar plated bacteria in a frequency of only 10^{-4} (see above).

In conclusion, mutant 811 was not able to cause severe pneumonia as the wild-type RC1 did in the animal host and showed a significantly reduced replication in comparison to the wild-type strain. Even though mutant 811 was completely serum sensitive, it was not cleared from the animal lung. Moreover, our data strongly indicate that two LPS phases of *L. pneumophila* are expressed in vivo.

Discussion

We here describe the isolation and investigation of an LPS-mutant of *Legionella pneumophila* SG 1, subgroup OLDA. In comparison to other Gram-negative bacteria, *L. pneumophila* exhibits an unusual LPS structure (31, 32, 34). The chemical structure has recently been analyzed for *L. pneumophila* SG 1, subgroup Philadelphia. It was found that the lipid A moiety consists of long-chain fatty acids which may account for its low endotoxic activity (31). The core oligosaccharide lacks heptose and phosphate groups and exhibits hydrophobic properties due to the presence of four *O*-acetyl groups and three deoxy-sugars (Rha 2, QuiNAc) (31, 34), which has so far not been found in any other bac-

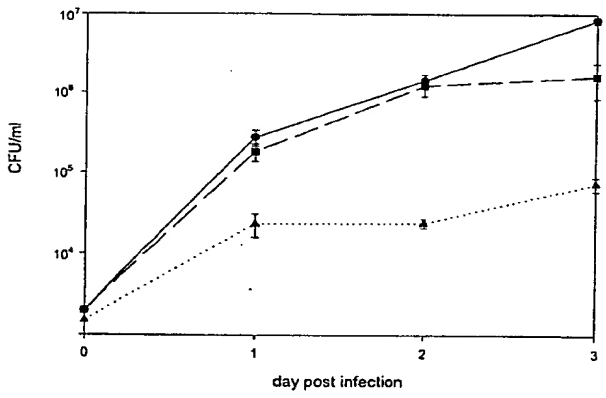

Figure 6. Infection of HL-60 cells with *L. pneumophila*. After 2 h coincubation of host cells and bacteria, extracellularly remaining bacteria were killed by gentamicin. The CFU determined at day 0 therefore represent intracellular bacteria. (*Circles*) Wild-type RC1; (*triangles*) mutant 811; (*squares*) 811-rev. CFU are shown as mean of duplicates.

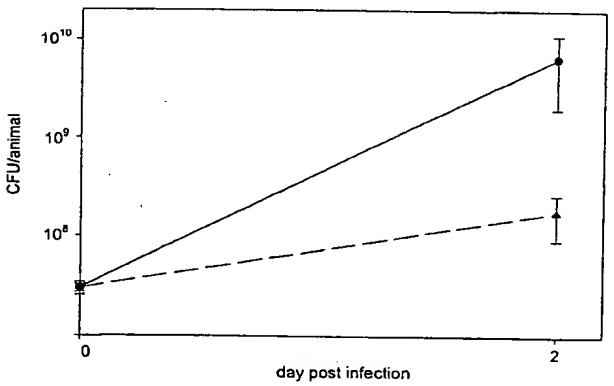

Figure 8. Investigation of virulence of *L. pneumophila* wild-type strain RC1 (*circle*) and mutant 811 (*triangle*) in the guinea pig animal model. Animals were intratracheally infected with 3×10^7 bacteria. Number of recovered bacteria was determined 48 h after infection by plating aliquots of lung suspensions.

terial strain. The O-chain is composed of an unbranched homopolymer with α-(2→4) interlinked 5-acetamidino-7-acetamido-8-*O*-acetyl-3,5,7,9-tetradeoxy-L-*glycero*-D-*galacto*-nonulosonic acid, termed legionaminic acid. Due to the lack of free hydroxyl groups and characteristic substituents, the O-chain is highly hydrophobic (31, 32). In this study we determined the chemical structure of the LPS O-antigen of *L. pneumophila* SG 1, subgroup OLDA. The O-chain was found to be of the same structure as the one from Philadelphia (31, 32), except that it lacks the 8-*O*-acetyl group and therefore is termed 8-*O*-deacetyl-legionaminic acid. The finding that OLDA strains all lack the 8-*O*-acetyl group was expected after the serological data gained by investigating antibody reactivities: the 8-*O*-acetyl group is known to be involved in binding of mAb 2 (27) and mAb 3/1 (46). In contrast, both antibodies do not bind to strains of the subgroup OLDA. The length of the O-chain of subgroup OLDA shows a bimodal distribution with maxima at 10–35 and 45–100 carbohydrate units, respectively. This banding pattern is very similar to that obtained for subgroup Philadelphia (32). LPS-mutant 811 did not show any differences in O-chain structure and length when compared with its parent wild-type strain RC1. Therefore, we conclude that LPS of mutant 811 is altered in the core oligosaccharide.

Mild acid hydrolysis in acetate buffer was used to cleave the lipid A moiety from the carbohydrate moiety of the LPS molecule. Under these conditions the ketosidic linkages of Kdo and iso-legionaminic acid are as well cleaved (32–34, 45), whereas *O*-acetyl groups remain intact (34, 45). It can be excluded that artifacts created by the acid hydrolysis treatment prevented the identification of modifications of the mutant 811 LPS compared with the wild-type LPS. Epitope mapping experiments showed that carbohydrate fractions isolated after mild acid hydrolysis and gel permeation chromatography still were able to compete for epitope binding by mAbs 2625 and LPS-1. If artifacts were generated by the degradation procedure, they did not interfere with those substituents important for formation of the epitopes bound by mAbs 2625 and LPS-1.

Our idea that the core sugar composition of mutant 811 differs from that of the parent wild-type RC1 is supported by the results of epitope mapping of mAb 2625 and mAb LPS-1. With the aid of a competition ELISA method we could show that mAb LPS-1 binds to the core oligosaccharide of *L. pneumophila* SG 1. mAb LPS-1 shows a stronger binding to mutant 811 than to wild-type RC1, indicating that alterations in the core structure of 811 enhance the accessibility of the LPS-1 epitope. In contrast, mAb 2625 binds to the LPS O-chain and the core oligosaccharide is presumably also involved in formation of the epitope. Moreover, short-chain polysaccharides showed a reduced binding to mAb 2625 in comparison to long-chain fractions. This finding supports our hypothesis that a distinct O-chain length is required for mAb 2625 reactivity.

In vitro and in vivo experiments revealed that virulence of mutant 811 was significantly reduced in comparison to the wild-type strain RC1. In in vitro assays, LPS-mutant 811 was unable to replicate intracellularly in macrophage-like cells even though the mutant bacteria were able to enter the host cells. In addition, mutant 811 was rapidly killed by serum complement factors, whereas the corresponding wild-type strain was almost resistant to complement lysis. By comparative analysis of LPS-mutant 811 and the parent wild-type RC1, these data show for the first time that resistance to serum complement of *L. pneumophila* is mediated by LPS carbohydrate moiety. However, we cannot exclude that the LPS variation of mutant 811 leads to additional modifications in the formation of the outer membrane. Such alterations could for example affect surface molecules which might as well be required for serum resistance and virulence of *L. pneumophila*. For many bacterial pathogens, surface polysaccharide structures such as capsules and LPS were found to be involved in complement inhibition (reviewed in references 47, 48). Currently we do not know whether killing of mutant 811 by serum complement factors is due to an increase in insertion of membrane attack complex (MAC) into the membrane of mutant 811 or if insertion of MAC occurs to the same extent in both, wild-type and mutant, but does not lyse wild-type cells. Therefore, future experiments should address the ability of wild-type RC1 and mutant 811 to prevent or promote insertion of MAC. In particular, activation of complement and deposition of complement factors C3b and C3bi on the cell surface is of special interest, since these molecules are known to mediate uptake into the host cells via complement receptors CR1 and CR3 (9, 10).

In the guinea pig animal model, mutant 811 was not able to cause severe pneumonia in guinea pigs infected with a dose that corresponds to the LD_{50} of wild-type strain RC1. Moreover, number of wild-type bacteria recovered from the lungs of infected animals exceeded the number of mutant bacteria recovered from infected animals by 1 to 2 orders of magnitude. Most interestingly, LPS-mutant 811 exhibited an unstable phenotype. The majority of cells from the originally isolated clone 811, which was negative for mAb 2625 binding, switched back to the wild-type phenotype and restored mAb 2625 binding. This phase variation between two LPS phenotypes was found to be immensely promoted in vivo in the animal host. Inoculated bacteria of mutant 811 included 8% cells positive for mAb 2625 binding of the wild-type phenotype. In contrast, among recovered bacteria from the infected animals the portion of wild-type bacteria was increased to 35%, indicating a selective advantage of the wild-type phenotype over the mutant phenotype. Bacteria recovered from animals infected with the wild-type RC1 contained 5% cells negative for mAb 2625 binding and therefore exhibiting the phenotype of mutant 811. We observed the same phase variation of wild-type RC1 and mutant 811 when the bacteria were incubated with heat inactivated human serum for 1 h (data not shown). Our results strongly indicate that phase variation of *L. pneumophila* LPS is induced and promoted by the animal host and by human serum. This is the first description of a phase-variable expression of surface polysaccharides of *L. pneumophila*. Diversity of surface carbohydrates

achieved by means of high-frequency, reversible switching of sugar epitopes has been described and intensively studied in *Haemophilus influenzae* (49–58), *Neisseria gonorrhoeae* (59, 60) and *N. meningitidis* (61–64). In *H. influenzae*, expression of enzymes involved in LPS biosynthesis is controlled by multiple repeats of tetrameric nucleotides within the *lic, lex2,* and *lgtC* loci (50, 56, 58). A change in the number of tetrameric repeats, arising through slipped-strand mispairing, results in a frame shift mutation, thus preventing expression of the encoded enzyme. Generation of phenotypic LPS variation by these intragenic alterations is considered as a virulence mechanism, enabling the bacteria to adapt to different environmental conditions (55). Essentially the same slipped-strand mispairing mechanism is found in *Neisseria*. Genes of the *lgt* locus, encoding glycosyl-transferases responsible for LPS biosynthesis, are expressed or not depending on the number of guanosine residues within a poly-G stretch in the coding sequence (59–61). In *N. meningitidis*, expression of the terminal lacto-*N*-neotetraose on the LPS, which requires the glycosyl-transferase encoded by *lgtA*, is correlated with serum resistance, non-invasiveness and predominance in the blood of infected mice. In contrast, strains with the lacto-*N*-neotetraose negative LPS phenotype are serum-sensitive, invasive and predominantly found in the nasopharynx (61, 62, 65, 66). For *L. pneumophila*, it remains to be established by which molecular mechanism LPS phase variation is determined. A prerequisite to further investigate this question is the characterization of genes involved in LPS biosynthesis. Except for an *O*-acetyl-transferase gene (Mintz, C.S., unpublished data, accession number U32118) such genes have not yet been identified in *Legionella*.

Species of the genus *Legionella* do not express a capsule or an exopolysaccharide. Therefore the LPS carbohydrate moiety is the predominant molecule on the cell surface of these bacteria which contributes to the cell surface properties in an exceptionally important way. Nothing is known about adherence of legionellae to the lung epithelium. The ability of *L. pneumophila* to replicate in alveolar epithelial cells has been reported (67, 68). It is conceivable that adhesion and tight attachment to epithelial cells is a crucial step in infection before the target host cell can be invaded. Attachment and adhesion could be mediated by surface carbohydrates such as LPS, which has been suggested to act as an adhesin of numerous pathogenic bacteria (reviewed in reference 69). Moreover, LPS of *L. pneumophila* may also be involved in attachment to its host cell. In a very recent study, a 170-kD lectin of *Hartmanella vermiformis* has been identified as a potential receptor used by *L. pneumophila* to invade the protozoan cell (70). However, the ligand on the bacterial surface remains to be identified. Future studies should therefore focus on the role of LPS in attachment and adhesion to different host cells and environments that are exploited by *L. pneumophila*.

We are grateful to Bettina Brand for help with the HL-60 infection assay. Larry Phillips is acknowledged for critical suggestions on the manuscript.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft to E. Lüneberg.

Address correspondence to Edeltraud Lüneberg, Institut für Hygiene und Mikrobiologie, Universität Würzburg, Josef-Schneider-Str. 2, 97080 Würzburg, Germany. Phone: 49-931-201-3936; Fax: 49-931-201-3445; E-mail: elueneberg@hygiene.uni-wuerzburg.de

Received for publication 5 December 1997 and in revised form 8 April 1998.

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